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| (54) Title: SENSITIZATION OF HER2/neu OVER-EXPRESSING CANCER CELLS TO CHEMOTHERAPEUTIC DRUGS | | | |
| (57) Abstract The present invention relates to methods for the inhibition of the gene product of the neu oncogene, p185neu tyrosine kinase. Over-expression of the neu oncogene leads to chemoresistance. The methods disclosed involve the novel use of emodin in combination with chemotherapeutic drugs to treat carcinoma. Furthermore, emodin surprisingly potentiates the antineoplastic effects of the chemotherapeutic agents. The inventors propose that emodin sensitizes cancer cells such that they become amenable to treatment by chemotherapeutic drugs. | | | |
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DESCRIPTION

SENSITIZATION OF HER2/*neu* OVEREXPRESSING CANCER CELLS TO CHEMOTHERAPEUTIC DRUGS

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BACKGROUND OF THE INVENTION

A. Field of the Invention

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The present invention relates to the treatment of cancer. In some aspects, this invention relates to the suppression of oncogenesis that is mediated by the HER-2/*neu* (*neu*) oncogene, an oncogene which has been correlated with many cancer types in humans. Methods and compositions for the treatment of *neu*-mediated cancer using drug combinations are disclosed.

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B. Background of the Related Art

Breast cancer remains a major cause of cancer death in women. It is estimated that 180,000 new cases of breast cancer are diagnosed and 46,000 women die of breast cancer in the United States alone over a period of one year. Hence, there is an urgent need to develop novel agents breast cancer diagnosis, prognosis prevention and therapy.

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Lung cancer is also a major cause of cancer death in the United States (Silverberg *et al.*, 1988). Lung cancers are usually divided into two groups by clinical and biological criteria: (1) non-small-cell lung cancer (NSCLC) (Boring *et al.*, 1994), and (2) small-cell lung cancer (SCLC). Most small-cell lung cancers are sensitive to chemotherapy, whereas NSCLC usually are refractory to chemotherapy at the time of

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- 2 -

diagnosis (Minna *et al.*, 1989). Thus NSCLC are the cause of most lung cancer deaths (Boring, *et al.*, 1994). To identify more effective therapeutic agents for lung cancer, intensive effort has been made to characterize specific gene alteration in lung cancers and to develop therapies that target those genes.

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It is well established that a variety of cancers are caused, at least in part, by genetic abnormalities that result in either the over-expression of one or more genes, or the expression of an abnormal or mutant gene or genes. For example, in many cases, the expression of oncogenes is known to result in the development of cancer.

10 "Oncogenes" are genetically altered genes whose mutated expression product somehow disrupts normal cellular function or control (Spandidos *et al.*, 1989).

Most oncogenes studied to date have been found to be "activated" as the result of a mutation, often a point mutation, in the coding region of a normal cellular gene, *i.e.*, a "proto-oncogene", that results in amino acid substitutions in the expressed protein product. This altered expression product exhibits an abnormal biological function that takes part in the neoplastic process (Travali *et al.*, 1990). The underlying mutations can arise by various means, such as by chemical mutagenesis or ionizing radiation. A number of oncogenes and oncogene families, including *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun* and *abl*, have now been identified and characterized to varying degrees (Travali *et al.*, 1990; Bishop, 1987).

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About one third of the known oncogenes encode proteins that phosphorylate tyrosine residues on other proteins. These enzymes are known as tyrosine specific protein kinases. The reaction they catalyze is the transfer of the terminal phosphoryl group of ATP to the hydroxyl group of a tyrosine residue in a protein. Included in this family of oncogenes are *src*, *yes*, *fps*, *fes*, *abl*, *ros*, *fgr*, *erbB*, *fms*, *mos*, *raf*, and *neu*.

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The *neu* gene (also known as *HER-2/neu* or *c-erb-2*) encodes a 185-kDa transmembrane tyrosine kinase (p185^{neu}) with homology to epidermal growth factor receptor (Hung *et al.*, 1986; Coussens *et al.*, 1985; Schechter *et al.*, 1984; Semba *et al.*, 1985; Yamamoto *et al.*, 1986). Enhanced expression of *neu* is known to be
5 involved in many human cancers, including NSCLC and has been shown to correlate with poor patient survival in NSCLC (Kern *et al.*, 1990; Schneider *et al.*, 1981; Weiner *et al.*, 1990). Cellular and animal studies have shown that an increase in *neu* tyrosine kinase activity increases the expression of malignant phenotypes (Muller *et al.*, 1988; Hudziak *et al.*, 1987; Muthuswamy *et al.*, 1994; Yu *et al.*, 1991; Yu *et al.*,
10 1993; Hung *et al.*, 1989; Sistonen *et al.*, 1989; Yu *et al.*, 1994).

The *neu* oncogene, was first identified in transfection studies in which NIH 3T3 cells were transfected with DNA from chemically induced rat neuroglioblastomas (Shih *et al.*, 1981). The p185 protein encoded by *neu* has an extracellular,
15 transmembrane, and intracellular domain, and therefore has a structure consistent with that of a growth factor receptor (Schechter *et al.*, 1984). The human *neu* gene was first isolated due to its homology with *v-erbB* and EGF-r probes (Semba *et al.*, 1985).

The *neu* oncogene plays an important role in carcinogenesis. The gene is
20 amplified in approximately 30% of primary breast cancer. Amplified expressions of the *neu* oncogene in transfected 3T3 cells induces malignant transformation.

Along with an increased proliferative potential, *neu*-mediated cancers appear to be resistant to host defense mechanisms. Studies have shown that overexpression
25 of the *neu* oncogene in transfected cells results in resistance to tumor necrosis factor, a major effector molecule in macrophage-mediated tumor cell cytotoxicity.

neu expression has also been detected in ovarian cancer and its overexpression results in poor prognosis. The expression of *neu* oncogenes in human tumor cells induce resistance to several host cytotoxic mechanisms.

5 Therefore, *neu* oncogene expression is correlated with the incidence of cancers of the human breast and female genital tract. Moreover, amplification/overexpression of this gene has been directly correlated with relapse and survival in human breast cancer (Slamon *et al.*, 1987; 1989). Therefore, it is important to evolve information regarding the *neu* oncogene, particularly information that could be applied to
10 reversing or suppressing the oncogenic progression that seems to be elicited by the presence or activation of this gene. Unfortunately, little has been previously known about the manner in which one may proceed to suppress the oncogenic phenotype associated with the presence of oncogenes such as the *neu* oncogene.

15 In addition, *neu* overexpression in NSCLC is associated with shortened survival. *In vitro* experimental models have provided evidence that, in the murine cell NIH 3T3, oncogenes increase drug resistance. Furthermore, Tsai *et al.*, 1993 and 1995 used a NSCLC model to demonstrate that activation of an oncogene is quantitatively associated with intrinsic chemoresistance in human malignant cells.
20 This resistance is observed with a variety of drugs that are structurally unrelated and act on different targets and/or by different mechanisms. Thus increased expression of *neu* oncogene enhances chemoresistance to a wide variety of chemotherapeutic agents (Tsai, 1993) including cisplatin, doxorubicin, and VP16 (Tsai *et al.*, 1993; Tsai *et al.*, 1995). The association of *neu* overexpression in cancer cells with malignant
25 phenotypes and chemoresistance provides a plausible interpretation for the poor clinical outcome for patients with *neu*-overexpressing tumors.

Although breast cancer diagnosed in its earliest clinical stages (stage 0, stage Ia) is highly curable, the cure rate for more advanced stages drops precipitously, even

after modern combined-modality treatments. Metastatic breast cancer responds to both chemotherapy and hormone therapy, and most patients can be palliated adequately during the 1 to 3 years of usual survival. However, metastatic breast cancer is considered incurable, as demonstrated by the relentless death rates, regardless of the treatment modality utilized. Front-line chemotherapy or hormone therapy programs for correctly selected patients produce objective responses in 50% to 70% of patients, but the median duration of response is usually less than one year. Response rates after second line treatments are considerably lower (20% to 50%), and response durations average 6 months.

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Ovarian cancer is also highly curable in its earliest stages, but the overwhelming majority of patients are diagnosed in stages III and IV. Although responsive to chemotherapy, most patients with advanced ovarian cancer relapse and die of their disease. With the introduction of several new cytotoxic agents (taxanes, vinorelbine, platinum derivatives), some responses are observed after second line therapy too, but cure in this situation remains an elusive goal.

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Therefore, there is an urgent need to develop novel anti-cancer agents for these types of cancer.

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SUMMARY OF THE INVENTION

The present invention seeks to overcome these and other drawbacks inherent in the prior art by administering an agent that sensitizes cancer cells to chemotherapeutic agents.

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Some embodiments of the invention involve methods of inhibiting transformation of a cell, in some particular embodiment, oncogene-mediated transformation of a cell. Generally, these methods comprise the step of contacting the

cell with an emodin-like tyrosine kinase inhibitor and a chemotherapeutic drug in amounts effective to inhibit the transformed phenotype. In a preferred embodiment, the transformation being inhibited will be *neu* oncogene-mediated transformation. Also, preferably, the embodiments in which transformation is to be inhibited will comprises a tyrosine specific protein kinase encoded by *neu*. Of course, the invention
5 also applies to methods of inhibiting other oncogene-mediated transformation events, such as transformation by *ras*, *src*, *yes*, *fps*, *fes*, *abl*, *ros*, *fgr*, *erbB*, *fms*, *mos*, *raf*, etc.

Emodin-like tyrosine kinase inhibitors of the invention are those compounds
10 that exhibit similar characteristics to those of emodin with regard to tyrosine kinase inhibition and the inhibition of *neu*-mediated transformation. Typical of such emodin-like tyrosine kinase inhibitors are anthraquinones that have a chemical structure identical or similar to those in Table 1. Of course the invention is not limited to the use of these inhibitors and other inhibitors that possess the structural
15 and/or functional properties of emodin may be used. In some preferred embodiments, the emodin-like tyrosine kinase inhibitor is an anthraquinone tyrosine kinase inhibitor. The emodin-like tyrosine kinase inhibitor may be, for example, emodin, emodin-8-O-D-glucoside, chrysophanic acid, gluco-chrysophanic acid, physcion, or physcion-8-O-D-glucoside or any of the other structures in Table 1 for example.
20 DK-III-8; DK-III-19; DK-III-47; DK-III-48; DK-III-13; DK-III-11; DK-II-1; DK-II-2; DK-IV-1; DK-V-47; DK-V-48; DK-III-52. In one preferred embodiment, the *neu* tyrosine kinase inhibitor is DK-V-47. In the most preferred embodiment the *neu* tyrosine kinase inhibitor is emodin.

25 In some embodiments of this invention a cell is contacted with between about 0.5mg/kg total weight and 500mg/kg total weight of the emodin-like tyrosine kinase inhibitor. In some particular embodiments, the cell is contacted with between about 0.5mg/kg total weight and 500mg/kg total weight of emodin. In still other embodiments the cell is contacted with between about 0.5mg/kg total weight and

500mg/kg total weight of an emodin-like tyrosine kinase inhibitor. In yet other embodiments the cell is contacted with between about 10 to about 100 μ M emodin, or between about 20 and 80 μ M, or between about 30 and 70 μ M; or between about 40 and 60 μ M or about 50 μ M emodin. . Total weight may be defined as the total weight of the cell or cells in culture, or the body weight of an animal, including a human.

Some embodiments of the invention involve chemotherapeutic agents. These are compounds that exhibit some form of anti-cancer activity. In some preferred embodiments, the chemotherapeutic drug is an alkylating agent, plant alkaloid, antibiotic, or antineoplastic agent. In those embodiments of the invention where the chemotherapeutic is an alkylating agent, the alkylating agent may be, for example, mechlorethamine, cyclophosphamide, ifosfamide, chlorambucil, melphalan, busulfan, thiopeta, carmustine, lomustine, and/or streptozin. In those embodiments where the chemotherapeutic agent comprises a plant alkaloid, the plant alkaloid is, for example, vincristine, vinblastine or taxol. In a preferred embodiment, the plant alkaloid is taxol. In those embodiments of the invention where the chemotherapeutic agent is an antibiotic, the antibiotic may be, for example, dactinomycin, daunorubicin, idarubicin, bleomycin, mitomycin or doxorubicin. In most preferred embodiments the antibiotic is doxorubicin. In other embodiments where the chemotherapeutic agent comprises an antineoplastic, the preferred antineoplastic is, for example, cisplatin, VP16 and TNF.

In certain embodiments of the invention, the emodin or emodin-like tyrosine kinase inhibitor is administered to the cell prior to the administration of the chemotherapeutic agent. In other aspects of the invention, the chemotherapeutic agent is administered to the cell prior to administration of the emodin or emodin-like inhibitor. Alternatively the emodin or emodin-like tyrosine kinase inhibitor and the chemotherapeutic drug are administered simultaneously.

In some embodiments of the invention, the cell is located within an animal and effective amounts of the emodin or emodin-like tyrosine kinase inhibitor and the chemotherapeutic drug are administered to the animal. In certain embodiments of the invention, the chemotherapeutic drug and the emodin or emodin-like tyrosine kinase inhibitor are suitably dispersed in a pharmacologically acceptable formulation. In certain preferred embodiments where the cell is an animal cell, the cell is a human cell. In other preferred embodiments the cell is a lung, cancer cell, ovarian cancer cell, or a breast cancer cell.

In some embodiments of the present invention the cell is contacted with a single composition comprising emodin or an emodin-like tyrosine kinase inhibitor in combination with a chemotherapeutic agent. In such cases, the composition may be suitably dispersed in a pharmacologically acceptable formulation.

The invention contemplates embodiments comprising sensitizing a cancer cell to a chemotherapeutic drug. These embodiments comprise exposing the cell with an effective amount of emodin or emodin-like. In some such embodiments inhibition of *neu*-mediated cancer is accomplished by administering an effective combination of emodin-like tyrosine kinase inhibitor and chemotherapeutic drug to an animal having or suspected of having cancer in an effective amount to inhibit the cancer. In embodiments where the composition is administered to an animal, the animal is typically a mammal. In such cases, the invention will be of particular use in the treatment and prevention of *neu*-mediated transformation in humans

Certain embodiments of the present invention comprise injecting a therapeutically effective amount of an emodin-like tyrosine kinase inhibitor into an animal and contacting the animal with a chemotherapeutic drug. In preferred embodiments the emodin-like tyrosine kinase inhibitor is an emodin. In certain

embodiments of the invention a cancer site is contacted with a chemotherapeutic drug by administering to the animal a therapeutically effective amount of a pharmaceutical composition comprising a chemotherapeutic drug wherein the chemotherapeutic drug is for example cisplatin, doxorubicin, VP16, taxol or TNF.

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The inventors have also enabled the production of pharmaceutical compositions comprising an emodin-like tyrosine kinase inhibitor and a chemotherapeutic drug in a pharmacological carrier. Those of skill will understand the nature of such pharmacological carriers based on the teachings of this specification and the current knowledge in the art. The pharmaceutical compositions of the invention may contain any of the emodin-like tyrosine kinase inhibitors and chemotherapeutic drugs mentioned above or elsewhere in this specification, or know to those of skill in the art. In a preferred pharmaceutical composition the chemotherapeutic drug is cisplatin, doxorubicin, etoposide, taxol or TNF. In some preferred embodiments, the emodin-like tyrosine kinase inhibitor is emodin.

The invention also encompasses pharmaceutical combinations comprising an emodin-like tyrosine kinase inhibitor and a chemotherapeutic drug. In certain preferred combinations, the tyrosine kinase inhibitor is emodin. The chemotherapeutic drug may be any that is listed elsewhere in this specification or known to those of skill in the art at the present or in the future. Exemplary chemotherapeutic drugs for us in the pharmaceutical combinations of the present invention are cisplatin, doxorubicin, etoposide, taxol and TNF. In certain embodiments of the invention the pharmaceutical combination may contain the emodin or emodin-like tyrosine kinase inhibitor and the chemotherapeutic drug within the same pharmaceutical composition.

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In other embodiments, the pharmaceutical combinations will comprise separate pharmaceutical compositions for each of the emodin or emodin-like tyrosine kinase inhibitor and the chemotherapeutic drug. These separate compositions may be combined internal to or external to a body to create the pharmaceutical combination.

Other embodiments of the invention include therapeutic kits comprising in suitable container, a pharmaceutical formulation of an emodin-like tyrosine kinase inhibitor, a pharmaceutical formulation of a chemotherapeutic drug, and/or a pharmaceutical formulation comprising both emodin or an emodin-like compound and a chemotherapeutic drug. The kit may also contain instructions on how to administer the pharmaceutical formulation or formulations of the kit to an animal either alone, or in combination with formulations that one may obtain separately from the kit. The kit may also comprise instructions that explain how to use the kit but are provided separately from the container of the kit. The kit may comprise the emodin or emodin-like tyrosine kinase inhibitor and chemotherapeutic drug to be present within a single container or alternatively the kit could comprise the emodin-like tyrosine kinase inhibitor and/or the chemotherapeutic drug are present within distinct containers.

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Some embodiments of the present invention relate to a method of inhibiting oncogene-mediated transformation of a cell, comprising contacting the cell with emodin or an emodin-like tyrosine kinase inhibitor. These compounds are well-described in this specification.. In preferred embodiments the oncogene-mediated transformation is *neu* oncogene-mediated transformation. Also, preferably, the embodiments in which transformation is to be inhibited will comprises a tyrosine specific protein kinase encoded by *neu*. The invention also contemplates pharmaceutical compositions, and kits comprising emodin or emodin-like tyrosine kinase inhibitors to inhibit *neu*-mediated transformation. Of course, the invention also applies to methods of inhibiting other oncogene-mediated transformation events, such as transformation by *ras*, *src*, *yes*, *fps*, *fes*, *abl*, *ros*, *fgr*, *erbB*, *fms*, *mos*, *raf*.

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BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A and FIG. 1B. Effect of dose dependence and time course of emodin treatment on tyrosine phosphorylation and expression of *neu* in human breast cancer MDA-MB453 cells. FIG. 1A, cells in the serum-free medium were incubated without (0) or with emodin (10 or 40 μ M) at 37°C for 24 h. FIG. 1B, cells were incubated without (-) treatment or with (+) emodin (40 μ M) at 37°C for different times. Cell extracts were immunoprecipitated by anti-p185^{neu} antibody (Anti-*neu*) and then western blotted with either antiphosphotyrosine antibody (Anti-PY) or anti-p185^{neu} antibody as described in Example 1.

FIG. 2A and FIG. 2B. Effect of emodin on tyrosine phosphorylation and expression of *neu* in human *neu* overexpressing breast cancer cells. Cells in the serum-free medium were incubated without (-) or with (+) 40 μ M emodin at 37°C for 24 h. and then cell lysates were immunoprecipitated by anti-p185^{neu} antibody and blotted with anti-phosphotyrosine (FIG. 2A) or with anti-p185^{neu} (FIG. 2B) antibodies as described in Example 1.

FIG. 3A and FIG. 3B. Effect of emodin on autophosphorylation and transphosphorylation of P185^{neu} in MDA-MB453 breast cancer cells. FIG. 3A show cells that were incubated without (lane 1) or with (lane 2) emodin (40 μ M) at 37°C for 24 h, then cell lysates (500 μ g) were immunoprecipitated and kinase activities were measured by incubation with [γ -³²P]ATP and enolase. Cell lysates

from untreated cells were immunoprecipitated, then incubated with [γ - 32 P]ATP, enolase and different concentrations of emodin for 20 minutes at room temperature (FIG. 3B). Reactants were resolved on 7.5% SDS-PAGE. The phosphorylation products were dried and visualized by autoradiography as described in Example 1.

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FIG. 4A and FIG. 4B. Effect of emodin on the proliferation of human breast cancer cells expressing different levels of *neu*. MDA-MB453, AU-565, BT-483 cells, which overexpress *neu* and MCF-7, MDA-MB231, HBL-100 cells, which express normal levels of *neu*, were incubated without or with different concentrations of emodin at 37°C for 72 h (FIG. 4A). The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment of emodin as 100%. MDA-MB453 cells were incubated without (control) or with emodin (40 μ M) at 37°C for different times, and cells were washed and counted by trypan blue exclusion with hemacytometer (FIG. 4B). All determinations were made in triplicate. Results are mean \pm SD.

FIG. 5. Effect of emodin on human breast cancer cell colony growth in soft agarose. Cells (1×10^3 cells/well) were seeded into 24-well plates in culture medium containing 0.35% agarose over a 0.7% agarose layer with or without 40 μ M emodin, and incubated for 3 weeks at 37°C. Colonies were stained with p-iodonitrotetrazolium violet and counted the percentage of colony formation was calculated by defining number of colonies in the absence of emodin as 100%. All determinations were made four times. Results are mean \pm SD.

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FIG. 6A and FIG. 6B. The effect of emodin on tyrosine phosphorylation and expression of *neu* in human lung cancer cells. FIG. 6A and FIG. 6B shows the effect of emodin on tyrosine phosphorylation and expression of *neu* in human lung

cancer cells. Cells were grown in serum-free medium were incubated without (-) or with (+) 30 μ M emodin at 37°C for 24 hours, and then cell lysates were separated by 6.5% SDS-PAGE and blotted with anti-phosphotyrosine (anti-PY) or with anti-p185^{neu} (anti-neu) antibodies as described in Example 1. FIG. 6A shows the effects of 30 μ M emodin on tyrosine phosphorylation in NCI-H1435 and NCI-H226. FIG. 6B shows the effects of 30 μ M emodin on tyrosine phosphorylation in NCI-H460, H460.neo, H460.eB2 and H460.eB3 cells

FIG. 7A and FIG. 7B. Effect of emodin on the proliferation of human lung cancer cells. Human lung cancer cells were incubated with or without different concentrations of emodin at 37°C for 72 hours. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment of emodin at 100%. All determinations were made in six replicates. Results are means \pm SD. FIG. 7A shows the effects of emodin on the proliferation of NCI-H1435 and NCI-H226 as measured by MTT assay. FIG. 7B shows the effects of emodin on the proliferation of NCI-H460, H460.neo, H460.eB2 and H460.eB3 as measured by MTT assay.

FIG. 8A, FIG. 8B and FIG. 8C. Effects of cisplatin, doxorubicin, and VP16 alone or in combination with emodin on the proliferation of human lung cancer cells. The effects on cell growth of NCI-H1435, H460.eB2, H460.eB3, NCI-H226, H460.neo, H460.eB2 and H460.eB3 cells were examined by MTT assay and the drug concentrations required to inhibit 50% of cell growth (IC₅₀) were calculated. Cells were incubated with the drugs at 37°C for 72 h. FIG. 8A shows the IC₅₀ for cisplatin in NCI-H1435, H460.eB2, H460.eB3, NCI-H226, H460.neo, H460.eB2 and H460.eB3 cells. FIG. 8B shows the IC₅₀ for doxorubicin in NCI-H1435, H460.eB2, H460.eB3, NCI-H226, H460.neo, H460.eB2 and H460.eB3 cells. FIG. 8C shows the IC₅₀ for VP16 in NCI-H1435, H460.eB2, H460.eB3, NCI-H226, H460.neo, H460.eB2 and H460.eB3 cells.

FIG. 9A, FIG. 9B, FIG. 9C, FIG. 9D, FIG. 9E, FIG. 9F, FIG. 9G and FIG. 9H. The effects of drug combinations on cell growth. All cells were treated with 30 μ M emodin alone or in combination with different drugs at 37°C for 72 h. The effect on cell growth were examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells not treated with emodin as 100%. The inhibitory effect was calculated by 100% minus percentage of cell proliferation. All determinations were made in six replicates. Results are means \pm SD.* Indicates synergism which were evaluated as described in Example 1. **FIG. 9A** shows the effects of drug treatment on NCI-H1435 cells these are 50 μ M cisplatin, 1.0 μ M doxorubicin or 1.0 μ M VP16. **FIG. 9B** shows the effects of drug treatment on NCI-H226 cells. These cells were treated with 5 μ M cisplatin, 0.5 μ M doxorubicin, or 0.1 μ M VP16. **FIG. 9C** shows the effects of drug treatment on NCI-H460. These cells were treated with 5 μ M cisplatin, 0.1 μ M doxorubicin, or 0.1 μ M VP16. **FIG. 9D** shows the effects of drug treatment on H460.neo. These cells were treated with 5 μ M cisplatin, 0.1 μ M doxorubicin, or 0.1 μ M VP16. **FIG. 9E** shows the effects of drug treatment on H460.eB2. These cells were treated with 75 μ M cisplatin, 0.5 μ M doxorubicin, or 0.5 μ M VP16. **FIG. 9F** shows the effects of drug treatment on H460.eB3. These cells were treated with 5 μ M cisplatin, 0.5 μ M doxorubicin, or 0.5 μ M VP16. **FIG. 9G** shows the effects of emodin, taxol and TNF α on MDA-MB361 human breast cancer cells. These cells were treated with 20 μ M emodin alone or in combination with 10nM taxol and 0.5nM TNF- α . **FIG. 9H** shows the effects of emodin, taxol and TNF- α on MDA-MB435 human breast cancer cells. These cells were treated with 20 μ M emodin alone or in combination with 0.1nM taxol and 0.1nM TNF- α .

FIG. 10A, FIG. 10B, FIG. 10C, FIG 10D, FIG. 10E AND FIG 10F. The effects of drug combinations on cell growth in soft agar. Cells (1×10^3 cells/well) were seeded into 24-well plates in culture medium containing 0.35% agarose over a 0.7% agarose layer. Colonies were stained with p-iodonitrotetrazolium violet and counted. The percentage of colony formation was calculated by defining number of colonies in the absence of emodin as 100%. All determinations were made four times. Results are mean \pm SD. FIG. 10A shows the effects of 30 μ M emodin alone or in combination with 5 μ M cisplatin, 0.1 μ M doxorubicin or 0.1 μ M VP16 on the cell growth of NCI-H460 cells. FIG. 10B shows the effect of 30 μ M emodin alone or in combination with 5 μ M cisplatin, 0.1 μ M doxorubicin or 0.1 μ M VP16 on the cell growth of NCI-H460.neo cells in soft agar. FIG. 10C shows the effect of 30 μ M emodin alone or in combination with 75 μ M cisplatin, 0.5 μ M doxorubicin or 0.5 μ M VP16 on the cell growth of H460.eB2 cells in soft agar. FIG. 10D shows the effect of 30 μ M emodin alone or in combination with 75 μ M cisplatin, 0.5 μ M doxorubicin or 0.5 μ M VP16 on the cell growth of H460.eB3 cells in soft agar. FIG. 10E shows the effect of 30 μ M emodin alone or in combination with 5 μ M cisplatin, 0.1 μ M doxorubicin or 0.1 μ M VP16 on the cell growth of NCI-H226 cells in soft agar. FIG. 10F shows the effect of 30 μ M emodin alone or in combination with 50 μ M cisplatin, 1 μ M doxorubicin or 1 μ M VP16 on the cell growth of NCI-H1435 cells in soft agar.

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FIG. 11. Effect of emodin on colony growth of human breast cancer cells (A) and activated *HER-2/neu* transformed 3T3 cells (B) in soft agarose. Cells (1×10^3 cells/well) were seeded into 24-well plates in culture medium containing 0.35% agarose over a 0.7% agarose layer with or without different concentrations of emodin or DK-V-47, and incubated for 3 weeks at 37°C. Colonies were stained with p-iodonitrotetrazolium violet and counted. The percentage of colony formation was

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calculated by defining number of colonies in the absence of emodin and DK-V-47 as 100%. All determinations were made four times. Results are mean \pm SD.

5 **FIG. 12. Effect of emodin and DK-V-47 on autophosphorylation and transphosphorylation of p185^{neu} in activated *HER-2/neu* transformed 3T3 cells.** Cell lysates from untreated cells were immunoprecipitated, then incubated with [γ -³²P]ATP, enolase and different concentrations of emodin and DK-V-47 for 20 min at room temperature. Reactants were resolved on 7.5% SDS-PAGE. The phosphorylation products were dried and visualized by autoradiography.

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FIG. 13. Effect of emodin on the proliferation of activated *HER-2/neu* transformed 3T3 cells. Cells were incubated without or with different concentrations of emodin or DK-V-47 at 37°C for 72 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the
15 absorption of cells without treatment of emodin and DK-V-47 as 100%. All determinations were made in triplicate. Results are mean \pm SD.

FIG. 14. Emodin and DK-V-47 reduce gelatinolytic activity of activated *HER-2/neu*-transformed 3T3 cells. Cells were treated or not treated with varying
20 concentrations of emodin and DK-V-47 overnight, the culture supernatants were collected and analyzed by zymography using gelatin-embedded SDS-PAGE. Gelatinolytic enzymes were detected as transparent bands on the blue-background of Coomassie blue-stained gels. Positions of the 92-kDa and 68-kDa gelatinase are indicated.

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FIG. 15. Emodin and DK-V-47 inhibit invasion of activated *HER-2/neu*-transformed 3T3 cells that have increased tyrosine kinase activity through a Matrigel layer. *In vitro* invasion was measured by using 24-well transwell

units with an 8-mm pore polycarbonate filter coated with Matrigel to form a thin, continuous layer on the filter top. The lower compartment contained 0.6 ml of laminin (20 mg/ml) as chemo-attractants. Activated *HER-2/neu*-transformed cells (5×10^4) were placed in the upper compartment and treated or not treated with emodin or DK-V-47 for 3 days. Then, lower surfaces of filters from the transwell units were fixed with 3% glutaraldehyde in PBS and stained with Giemsa solution. Chemo-invasive activity was determined under the microscope by counting cells that had migrated to the lower side of the filter. All experiments were done in triplicate.

FIG. 16A and FIG. 16B. Effect of taxol alone or in combination with emodin on the human breast cancer cell colony growth in soft agarose. Cells (1×10^3 cells/well) were seeded into 24-well plates in culture medium containing 0.35% agarose over a 0.7% agarose layer with or without either taxol or emodin alone, or emodin plus taxol and incubated for 3 weeks at 37°C . All cells were treated with 20 μM emodin alone or in combination with taxol. The doses of taxol used to treated different cancer cells are 1 μM for MDA-MB 361 cells (FIG. 16A); 0.1 nM for MDA-MB 435 cells (FIG. 16B). Colonies were stained with p-iodonitrotetrazolium violet and counted, and the percentage of colony formation was calculated by defining the number of colonies in the absence of drugs as 100%. All determinations were made four times. Bars, SD.

FIG. 17A and FIG. 17B. FIG. 17A. Tumor growth in mice bearing *HER-2/neu*-overexpressing breast cancer ($n = 10$). *HER-2/neu*-overexpressing MDA-MB361 cells (5×10^7) were injected into female nu/nu mice subcutaneously. Three weeks later, when the palpable solid tumors were detected. The mice were give either placebo, emodin (40 mg/kg body weight) or Taxol (10 mg/kg body weight), or Emodin (40 mg/kg) plus taxol (10 mg/kg) by i.p. injection twice a week for 8 weeks. The tumor volume was monitored weekly for 8 weeks. Mice were observed for

survival for up to 300 days. FIG. 17B. Survival in mice bearing *HER-2/neu*-overexpressing breast cancer.

DETAILED DESCRIPTION THE PREFERRED EMBODIMENTS

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The present invention seeks to overcome drawbacks inherent in the prior art by providing new treatment methods, compositions and kits for increasing the efficacy of antineoplastic agents against cancer

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The invention provides methods for treating cancers using emodin and/or emodin-like compounds that inhibit *neu*-tyrosine kinase activity. The methods of the invention generally rest in using emodin and/or emodin-like compound alone or in combination with another anti-cancer agent effective to treat the cancer cells.

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In one preferred embodiment, the inventors have discovered that the use of emodin, an exemplary emodin-like tyrosine kinase inhibitor, in combination with antineoplastic agents has a synergistic effect in inhibiting the growth of *neu*-mediated cancers, which are usually chemoresistant.

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The methods, compositions and kits of the invention may be used in conjunction with any emodin-like drug that has an active species or metabolite that, at least in part, inhibits protein tyrosine kinase activity. Such emodin-like inhibitors may themselves be the "active species". Emodin, chrysophanic acid, physcion and the glucosides of these compounds amongst others, are examples of this group of

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emodin-like inhibitor analogues. Many emodin-like inhibitors are, like emodin, anthraquinones. Alternatively, the emodin-like compound or drug may be one that exhibits similar properties to emodin in relation to *neu*-mediated cancer or is metabolized within the body to provide an active species or metabolite.

Structural Properties of Emodin and Anthraquinone-Based Emodin-Like Compounds

Emodin (3-methyl-1, 6, 8 trihydroxyanthra-quinone) belongs to a group of compounds that are structurally based upon the structure of anthraquinone shown in Table 1, to which various R groups may be added. A wide variety of anthraquinones exist in nature (Yeh *et al.*, 1988; Kupchan and Karim, 1976; Jayasuriya *et al.*, 1992). Structure B in Table 1 is emodin itself; C is emodin-8-O-D-glucoside; D is chrysophanic acid; E is gluco-chrysophanic acid; F is physcion; and G is physcion-8-O-D-glucoside. The emodin-like compounds of structures A, C and D-G are only exemplary forms of emodin-like compounds that may be used in the present invention. Numerous other emodin analogues are available as shown in Table 1 and as described by, *e.g.*, Yeh *et al.*, 1988; Kupchan and Karim, 1976; Jayasuriya *et al.*, 1992. Further the inventors have investigated additional emodin-like compounds recently developed by the research group of Ching-Jer Chang at Purdue University, West Lafayette and initial indications are that these compounds perform as well as or even better than emodin itself.

The first group (group A: Table 1 and Table 2) is comprised of compounds which structurally related to emodin, only replace CH₃ group with different other group at C₃ of emodin, their inhibitory activities of tyrosine phosphorylation of p185^{neu} are in following order CH₃ > C=NOCH₃ > CHNOH > CH₂OH > CONH₂ > COOH and inhibitory activities for proliferation of cells are CH₃ > CHNOH > CONH₂ > C=NOCH₃ > CH₂OH > CH₂OH > COOH, these results indicate that CH₃ group at C₃ position of emodin is very important to remain inhibitory activities of emodin on tyrosine phosphorylation and proliferation.

The second group (Group B: Table 1 and Table 2) also structurally related to emodin, only replace OH group with either H or OCH₃ group at C₆ position of

emodin. However, compare with emodin, their inhibitory activity for both tyrosine phosphorylation of p185^{neu} and proliferation of cells are 5-fold lower than emodin.

5 The third (Group C), after removed OH groups at C₁, C₆ and C₈, and CH₃ group at C₃ of emodin, added NH₂ group at C₁ and C₂ of emodin, decrease activity of emodin. The fifth group (group E) remove ketone group from C10, also reduce activity of emodin.

10 The fourth group (group D), structurally similar to the third group, except replace C₉ ketone with either p-acetylamidebenzomethyl group (DK-V-47) or p-aminobenzomethyl group (DK-V-48), DK-V-47 has higher activity than emodin to inhibit tyrosine phosphorylation of p185^{neu} and proliferation of cancer cells, however, replace COCH₃ of DK-V-47 with H group (DK-V-48), DK-V-48 decrease the activity of DK-V-47. These results suggest that COCH₃ group of DK-V-47 is
15 important to keep the activity of DK-V-47.

TABLE 1
Structures of Emodin-like Compounds

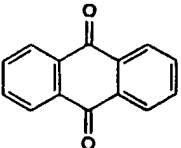
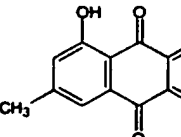
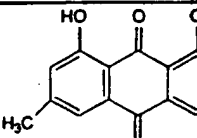
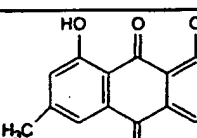
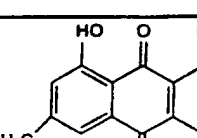
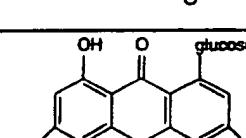
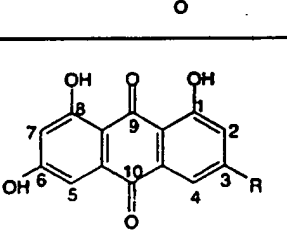
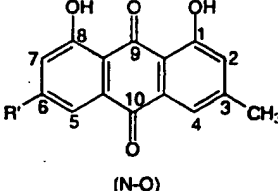
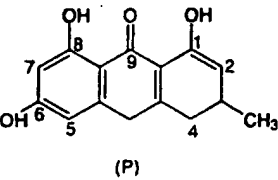
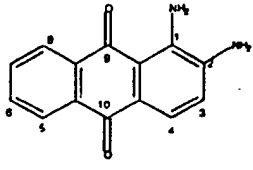
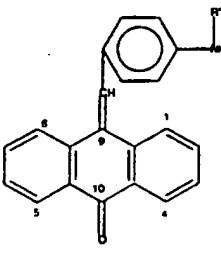
| | | |
|--|---|---|
|  | A. ANTHRAQUINONE | |
|  | B. EMODIN | |
|  | D. chrysophanic acid | |
|  | E. gluco-chrysophanic acid | |
|  | F. physcion | |
|  | G. physcion-8-O-D glucoside | |
|  <p>(H-M)</p> | Compounds | R |
| | | |
| | | |
| | | |
| | | |
| | | |
| | H-DK-III-8 I-DK-III-19 J-DK-III-47 K-DK-III-48 L-DK-III-13 M-DK-III-11 | -CH ₂ OH -CONH ₂ -C=NOCH ₃ -CNOH -COH -COOH |

TABLE 1, Continued

|  | Compounds | R' |
|---|------------------------|---------------------------------|
| (N-O) | N-DK-II-1 O-DK-II-2 | -H -OCH ₃ |
|  | P-DK-III-52 | |
| (P) | | |
|  | Compounds | R |
| (O) | O-DK-IV-1 | |
|  | R-DK-V-47 S-DK-V-48 | R' -CH ₂ CO -H |
| (R, S) | | |

5 Functional Properties of Emodin and Emodin-Like Compounds

Emodin, which was first isolated from *polygonum cuspidatum*, has been shown to be an inhibitor of the protein tyrosine kinase p56^{lck} (Jayasuriya *et al.*; 1992).

In the present invention, emodin is shown to inhibit *neu* tyrosine kinase activity and to preferentially repress the transformation ability and growth rate of

neu-overexpressing breast cancer cells. Emodin has been reported to be a tyrosine kinase inhibitor that restricts the activity of p56^{lck} kinase by preventing the binding of ATP *in vitro* (Jayasuriya *et al.*, 1992). Emodin also can inhibit the growth of cancer cells, including lymphocytic leukemia (Kupchan *et al.*, 1976), HL-60 human leukemia cells (Yeh *et al.*, 1988), and *ras*-transformed human bronchial epithelial cells (Chan *et al.*, 1993), by an unknown mechanism.

The inventors have found that emodin and emodin-like compounds assist in overcoming the chemoresistance of *neu*-overexpressing cancer cells by sensitizing such cells to chemotherapeutic agents. Having examined the effects of emodin on the tyrosine phosphorylation, proliferation and morphology, the inventors have examined the effect of emodin on tyrosine phosphorylation of *neu* in cancer cells, and tested the effects of combinations of emodin with chemotherapeutic agents on proliferation of these cells. The inventors found that emodin suppressed tyrosine phosphorylation of *neu*, preferentially inhibited proliferation of *neu*-expressing lung cancer cells to a surprising level, and sensitized these cells to chemotherapeutic drugs. This suppression of tyrosine phosphorylation is a functional characteristic of emodin-like compounds.

The inventors have demonstrated that emodin and emodin-like compounds suppress the tyrosine kinase activity of *neu*-overexpressing human breast cancer cells, suppresses their transforming ability, and induces their differentiation. Further, the inventors find that emodin also suppresses tyrosine phosphorylation of *neu* in lung cancer cells and preferentially inhibits growth of these cells. Further, this invention demonstrates that emodin is able to sensitize lung cancer cells that overexpress *neu* to the chemotherapeutic agents cisplatin, doxorubicin, and VP16. These results suggest that the tyrosine kinase activity of p185^{neu} is required for the chemoresistant phenotype of *neu* overexpressing cancer cells. Therefore, the invention shows that adding emodin to chemotherapeutic regimens greatly improves their efficacy.

Therapeutically Effective Amounts of Emodin and Emodin-Like Compounds

A therapeutically effective amount of an emodin-like tyrosine kinase inhibitor that is combined with a second agent as treatment varies depending upon the host treated and the particular mode of administration. In one embodiment of the invention the dose range of an emodin-like tyrosine kinase inhibitor used will be about 0.5mg/kg body weight to about 500mg/kg body weight. The term "body weight" is applicable when an animal is being treated. When isolated cells are being treated, "body weight" as used herein should read to mean "total cell weight". The term "total weight" may be used to apply to both isolated cell and animal treatment. All concentrations and treatment levels are expressed as "body weight" or simply "kg" in this application are also considered to cover the analogous "total cell weight" and "total weight" concentrations. However, those of skill will recognize the utility of a variety of dosage range, for example, 1mg/kg body weight to 450mg/kg body weight, 2mg/kg body weight to 400mg/kg body weight, 3mg/kg body weight to 350mg/kg body weight, 4mg/kg body weight to 300mg/kg body weight, 5mg/kg body weight to 250mg/kg body weight, 6mg/kg body weight to 200mg/kg body weight, 7mg/kg body weight to 150mg/kg body weight, 8mg/kg body weight to 100mg/kg body weight, 9mg/kg body weight to 50mg/kg body weight. Further, those of skill will recognize that a variety of different dosage levels will be of use, for example, 1mg/kg, 2mg/kg, 3mg/kg, 4mg/kg, 5mg/kg, 7.5mg/kg, 10, mg/kg, 12.5mg/kg, 15mg/kg, 17.5mg/kg, 20mg/kg, 25mg/kg, 30mg/kg, 35mg/kg, 40mg/kg, 45 mg/kg, 50mg/kg, 60mg/kg, 70mg/kg, 80mg/kg, 90mg/kg, 100mg/kg, 120mg/kg, 140mg/kg, 150mg/kg, 160mg/kg, 180mg/kg, 200mg/kg, 225 mg/kg, 250mg/kg, 275mg/kg, 300mg/kg, 325mg/kg, 350mg/kg, 375mg/kg, 400mg/kg, 450mg/kg, 500mg/kg, 550mg/kg, 600mg/kg, 700mg/kg, 750mg/kg, 800mg/kg, 900mg/kg, 1000mg/kg, 1250mg/kg, 1500mg/kg, 1750mg/kg, 2000mg/kg, 2500mg/kg, and/or 3000mg/kg. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be

of use in the invention, as are any ranges of dose defined by any two of these points. Any of the above dosage ranges or dosage levels may be employed for emodin alone or for emodin in combination with an anti-cancer drug.

5 "Therapeutically effective amounts" are those amounts effective to produce beneficial results in the recipient animal or patient. Such amounts may be initially determined by reviewing the published literature, by conducting *in vitro* tests or by conducting metabolic studies in healthy experimental animals. Before use in a clinical setting, it may be beneficial to conduct confirmatory studies in an animal model, 10 preferably a widely accepted animal model of the particular disease to be treated. Preferred animal models for use in certain embodiments are rodent models, which are preferred because they are economical to use and, particularly, because the results gained are widely accepted as predictive of clinical value.

15 As is well known in the art, a specific dose level of active compounds such as emodin and emodin-like compounds for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing 20 therapy. The person responsible for administration will determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

25 A composition of the present invention is typically administered orally or parenterally in dosage unit formulations containing standard, well known non-toxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parental as used herein includes subcutaneous injections, intravenous, intramuscular, intra-arterial injection, or infusion techniques.

In some embodiments, the emodin or emodin-like compound will be administered in combination with a second agent. So long as a dose of second agent that does not exceed previously quoted toxicity levels is not required, the effective amounts of the second agents may simply be defined as those amounts effective to reduce the cancer growth when administered to an animal in combination with the emodin-like agents. This is easily determined by monitoring the animal or patient and measuring those physical and biochemical parameters of health and disease that are indicative of the success of a given treatment. Such methods are routine in animal testing and clinical practice.

Examples of second agents that may be used with emodin or emodin-like tyrosine kinase inhibitor are anti-neoplastic agents. Examples of these are cisplatin; doxorubicin (Mechetner & Roninson, 1992) and analogues, such as 14-*O*-hemiesters of doxorubicin; etoposide; vincristine (Shirai *et al.*, 1994; Friche *et al.*, 1993); vinblastine (Bear, 1994; McKinney & Hosford, 1993); actinomycin D (McKinney & Hosford, 1993); daunomycin (Bear, 1994); daunorubicin (Muller *et al.*, 1994); taxotere (Hunter *et al.*, 1993); taxol (Mechetner & Roninson, 1992); and tamoxifen (Trump *et al.*, 1992). The skilled artisan is directed to "Physicians Desk Reference" 15th Edition, for dose ranges of chemotherapeutic agents practiced in the art. Some variation in dosage will necessarily occur depending on the condition of the subject being treated.

The treatment methods generally comprise administering to an animal with cancer, including a human patient, a therapeutically effective combination of emodin and or emodin-like tyrosine kinase inhibitor alone or in combination with one or more second agents that is effective in treating *neu*-mediated cancer growth exemplified by a decrease in the activity of *neu*-protein tyrosine kinase which is over-expressed in

neu-mediated cancers. The second agent(s) may be any of those listed above, and their functional equivalents.

Assays for Additional Emodin-Like Tyrosine Kinase Inhibitors for Use in the 5 Invention

10 In certain embodiments, the present invention concerns a method for identifying further *neu* protein tyrosine kinase inhibitors, which are "emodin-like compounds." It is contemplated that this screening technique will prove useful in the general identification of any compound that will serve the purpose of inhibiting *neu*-protein tyrosine kinase in a manner similar to the exemplary emodin-like tyrosine kinase inhibitors.

15 Useful compounds in this regard will not be limited to emodin. In fact, it may prove to be the case that the most useful pharmacological compounds for identification through application of the screening assay will be compounds that are structurally related to emodin. The active compounds may include fragments or parts of naturally-occurring compounds or may be only found as active combinations of known compounds which are otherwise inactive. However, prior to testing of such
20 compounds in humans or animal models, it will possibly be necessary to test a variety of candidates to determine which have potential.

25 Accordingly, in screening assays to identify pharmaceutical agents which inhibit *neu*-protein tyrosine kinase activity, it is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds.

In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to inhibit a tyrosine kinase assay, the method including generally the steps of:

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(a) obtaining an enzyme composition comprising a tyrosine kinase, preferably *neu*-tyrosine kinase that is capable of phosphorylating tyrosine;

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(b) admixing a candidate substance with the enzyme composition; and

(c) determining the ability of the candidate substance to inhibit tyrosine phosphorylation.

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To identify a candidate substance as being capable of inhibiting protein phosphorylation, one would first obtain an enzyme composition that is capable of phosphorylating tyrosine residues on a protein of interest. Naturally, one would measure or determine the phosphorylation activity of the tyrosine kinase composition in the absence of the added candidate substance. One would then add the candidate substance to the tyrosine kinase composition and re-determine the ability of the

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tyrosine kinase composition to phosphorylate tyrosine residues on a test protein in the presence of the candidate substance. A candidate substance which reduces the phosphorylation activity of the tyrosine kinase composition relative to the activity in its absence is indicative of a candidate substance with inhibitor capability.

25

The candidate screening assay is quite simple to set up and perform, and is related in many ways to the assay discussed above for determining enzyme activity. Thus, after obtaining a relatively purified preparation of the enzyme, either from native or recombinant sources, one will admix a candidate substance with the enzyme preparation, under conditions which would allow the enzyme to perform its tyrosine

phosphorylation function but for inclusion of a inhibitor substance. In this fashion, one can measure the ability of the candidate substance to reduce the tyrosine phosphorylation activity relatively in the presence of the candidate substance.

5 "Effective amounts" in certain circumstances are those amounts effective to reproducibly reduce *neu*-tyrosine kinase activity, or to reduce the growth of *neu*-mediated cancer cells, in comparison to their normal levels. Compounds that achieve significant appropriate changes in activity will be used. If desired, a battery of compounds may be screened *in vitro* to identify second agents for use in the present
10 invention.

 Significant decrease in tyrosine phosphorylation, *e.g.*, as measured using immunoblotting techniques with anti-phosphorylation antibodies, are represented by a reduction in protein phosphorylation levels of at least about 30%-40%, and most
15 preferably, by decreases of at least about 50%, with higher values of course being possible. Tyrosine kinase assays that measure tyrosine phosphorylation are well known in the art and may be conducted *in vitro* or *in vivo*.

 Inhibition of growth of cancer cells can measured by the MTT assay. A
20 significant inhibition in growth is represented by decreases of at least about 30%-40% as compared to uninhibited, and most preferably, of at least about 50%, with more significant decreases also being possible. Growth assays as measured by the MTT assay are well known in the art. Assays may be conducted as described by Mosmann *et al.*, 1983; Rubinstein *et al.*, 1990 (incorporated herein by reference). Therefore, if a
25 candidate substance exhibited inhibition in this type of study, it would likely be a suitable compound for use in the present invention.

 Quantitative *in vitro* testing is not a requirement of the invention as it is generally envisioned that the second agents will often be selected on the basis of their

known properties or by structural and/or functional comparison to those agents disclosed herein. Therefore, the effective amounts will often be those amounts proposed to be safe for administration to animals in another context, for example, as disclosed herein. As the invention arises in part from the inventors' discovery of certain metabolic and physiological events, and the inventors' surprising combination of elements, there is considerable information available on the use and doses of second agents alone, which information may now be employed with the present invention.

10 It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

15 In still further embodiments, the present invention is concerned with a method of inhibiting *neu*-tyrosine kinase activity which includes subjecting the enzyme to an effective concentration of an emodin-like inhibitor such as one of the family of compounds discussed above, or with a candidate substance identified in accordance with the candidate screening assay embodiments. This is, of course, an important aspect of the invention in that it is believed that by inhibiting the *neu* tyrosine kinase activity, one will be enabled to treat various aspects *neu*-mediated cancers caused by over-expression of *neu*. The use of such emodin-like inhibitors to block *neu*-tyrosine kinase activity will serve to treat *neu*-mediated cancers. The inventors have found that *neu*-mediated cancer cells, which are typically chemoresistant may be sensitized to 20 chemotherapeutic agents. As such emodin-like tyrosine kinase inhibitors are useful in conjunction with other chemotherapeutic therapies.

B. Chemotherapeutic Agents

5 A wide variety of chemotherapeutic agents may be used in combination with the emodin or emodin-like tyrosine kinase inhibitors of the present invention. These can be, for example, agents that directly cross-link DNA, agents that intercalate into DNA, and agents that lead to chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

10 Agents that directly cross-link nucleic acids, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic antineoplastic combination. Agents such as cisplatin, and other DNA alkylating agents may be used.

15 Agents that damage DNA also include compounds that interfere with DNA replication, mitosis, and chromosomal segregation. Examples of these compounds include adriamycin (also known as doxorubicin), VP-16 (also known as etoposide), verapamil, podophyllotoxin, and the like. Widely used in clinical setting for the treatment of neoplasms these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m² at 21 day intervals for adriamycin.
20 to 35-100 mg/m² for etoposide intravenously or orally.

Antibiotics

Doxorubicin

25 Doxorubicin hydrochloride, 5,12-Naphthacenedione, (8*s-cis*)-10-[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-hydrochloride (hydroxydaunorubicin hydrochloride, Adriamycin) is used in a wide antineoplastic spectrum. It binds to DNA and inhibits nucleic acid synthesis, inhibits mitosis and promotes chromosomal aberrations.

Administered alone, it is the drug of first choice for the treatment of thyroid adenoma and primary hepatocellular carcinoma. It is a component of 31 first-choice combinations for the treatment of ovarian, endometrial and breast tumors, bronchogenic oat-cell carcinoma, non-small cell lung carcinoma, gastric adenocarcinoma, retinoblastoma, neuroblastoma, mycosis fungoides, pancreatic carcinoma, prostatic carcinoma, bladder carcinoma, myeloma, diffuse histiocytic lymphoma, Wilms' tumor, Hodgkin's disease, adrenal tumors, osteogenic sarcoma soft tissue sarcoma, Ewing's sarcoma, rhabdomyosarcoma and acute lymphocytic leukemia. It is an alternative drug for the treatment of islet cell, cervical, testicular and adrenocortical cancers. It is also an immunosuppressant.

Doxorubicin is absorbed poorly and typically is administered intravenously. The pharmacokinetics are multicompartmental. Distribution phases have half-lives of 12 minutes and 3.3 hr. The elimination half-life is about 30 hr. Forty to 50% is secreted into the bile. Most of the remainder is metabolized in the liver, partly to an active metabolite (doxorubicinol), but a few percent is excreted into the urine. In the presence of liver impairment, the dose should be reduced.

Appropriate doses are, intravenous, adult, 60 to 75 mg/m² at 21-day intervals or 25 to 30 mg/m² on each of 2 or 3 successive days repeated at 3- or 4-wk intervals or 20 mg/m² once a week. The lowest dose should be used in elderly patients, when there is prior bone-marrow depression caused by prior chemotherapy or neoplastic marrow invasion, or when the drug is combined with other myelopoietic suppressant drugs. The dose should be reduced by 50% if the serum bilirubin lies between 1.2 and 3 mg/dL and by 75% if above 3 mg/dL. The lifetime total dose should not exceed 550 mg/m² in patients with normal heart function and 400 mg/m² in persons having received mediastinal irradiation. Alternatively, 30 mg/m² on each of 3 consecutive days, repeated every 4 wk. Exemplary doses may be 10 mg/m², 20 mg/m², 30 mg/m², 50 mg/m², 100 mg/m², 150 mg/m², 175 mg/m², 200 mg/m², 225 mg/m², 250 mg/m².

275 mg/m², 300 mg/m², 350 mg/m², 400 mg/m², 425 mg/m², 450 mg/m², 475 mg/m², 500 mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

5 In the present invention the inventors have employed emodin as an exemplary emodin-like inhibitor to synergistically enhance the antineoplastic effects of the doxorubicin in the treatment of cancers. Those of skill in the art will be able to use the invention as exemplified potentiate the effects of doxorubicin in a range of different *neu*-mediated cancers.

10

Daunorubicin

Daunorubicin hydrochloride, 5.12-Naphthacenedione, (8*S-cis*)-8-acetyl-10-
[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexanopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-
trihydroxy-10-methoxy-, hydrochloride; also termed cerubidine and available from
15 Wyeth. Daunorubicin intercalates into DNA, blocks DAN-directed RNA polymerase
and inhibits DNA synthesis. It can prevent cell division in doses that do not interfere
with nucleic acid synthesis.

In combination with other drugs it is included in the first-choice chemotherapy
20 of acute myelocytic leukemia in adults (for induction of remission), acute lymphocytic
leukemia and the acute phase of chronic myelocytic leukemia. Oral absorption is
poor, and it must be given intravenously. The half-life of distribution is 45 minutes
and of elimination, about 19 hr. The half-life of its active metabolite, daunorubicinol,
is about 27 hr. Daunorubicin is metabolized mostly in the liver and also secreted into
25 the bile (ca 40%). Dosage must be reduced in liver or renal insufficiencies.

Suitable doses are (base equivalent), intravenous adult, younger than 60 yr. 45
mg/m²/day (30 mg/m² for patients older than 60 yr.) for 1, 2 or 3 days every 3 or 4 wk
or 0.8 mg/kg/day for 3 to 6 days every 3 or 4 wk; no more than 550 mg/m² should be

given in a lifetime, except only 450 mg/m² if there has been chest irradiation; children, 25 mg/m² once a week unless the age is less than 2 yr. or the body surface less than 0.5 m. in which case the weight-based adult schedule is used. It is available in injectable dosage forms (base equivalent) 20 mg (as the base equivalent to 21.4 mg of the hydrochloride). Exemplary doses may be 10 mg/m², 20 mg/m², 30 mg/m², 50 mg/m², 100 mg/m², 150 mg/m², 175 mg/m², 200 mg/m², 225 mg/m², 250 mg/m², 275 mg/m², 300 mg/m², 350 mg/m², 400 mg/m², 425 mg/m², 450 mg/m², 475 mg/m², 500 mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

10

Mitomycin

Mitomycin (also known as mutamycin and/or mitomycin-C) is an antibiotic isolated from the broth of *Streptomyces caespitosus* which has been shown to have antitumor activity. The compound is heat stable, has a high melting point, and is freely soluble in organic solvents.

15

Mitomycin selectively inhibits the synthesis of deoxyribonucleic acid (DNA). The guanine and cytosine content correlates with the degree of mitomycin-induced cross-linking. At high concentrations of the drug, cellular RNA and protein synthesis are also suppressed.

20

In humans, mitomycin is rapidly cleared from the serum after intravenous administration. Time required to reduce the serum concentration by 50% after a 30 mg. bolus injection is 17 minutes. After injection of 30 mg., 20 mg., or 10 mg. I.V., the maximal serum concentrations were 2.4 mg./mL, 1.7 mg./mL, and 0.52 mg./mL, respectively. Clearance is effected primarily by metabolism in the liver, but metabolism occurs in other tissues as well. The rate of clearance is inversely proportional to the maximal serum concentration because, it is thought, of saturation of the degradative pathways.

25

Approximately 10% of a dose of mitomycin is excreted unchanged in the urine. Since metabolic pathways are saturated at relatively low doses, the percent of a dose excreted in urine increases with increasing dose. In children, excretion of intravenously administered mitomycin is similar.

Actinomycin D

Actinomycin D (Dactinomycin) [50-76-0]; $C_{62}H_{86}N_{12}O_{16}$ (1255.43) is an antineoplastic drug that inhibits DNA-dependent RNA polymerase. It is a component of first-choice combinations for treatment of choriocarcinoma, embryonal rhabdomyosarcoma, testicular tumor and Wilms' tumor. Tumors which fail to respond to systemic treatment sometimes respond to local perfusion. Dactinomycin potentiates radiotherapy. It is a secondary (efferent) immunosuppressive.

Actinomycin D is used in combination with primary surgery, radiotherapy, and other drugs, particularly vincristine and cyclophosphamide. Antineoplastic activity has also been noted in Ewing's tumor, Kaposi's sarcoma, and soft-tissue sarcomas. Dactinomycin can be effective in women with advanced cases of choriocarcinoma. It also produces consistent responses in combination with chlorambucil and methotrexate in patients with metastatic testicular carcinomas. A response may sometimes be observed in patients with Hodgkin's disease and non-Hodgkin's lymphomas. Dactinomycin has also been used to inhibit immunological responses, particularly the rejection of renal transplants.

Half of the dose is excreted intact into the bile and 10% into the urine; the half-life is about 36 hr. The drug does not pass the blood-brain barrier. Actinomycin D is supplied as a lyophilized powder (0/5 mg in each vial). The usual daily dose is 10 to 15 mg/kg; this is given intravenously for 5 days; if no manifestations of toxicity are encountered, additional courses may be given at intervals of 3 to 4 weeks. Daily

injections of 100 to 400 mg have been given to children for 10 to 14 days: in other regimens, 3 to 6 mg/kg, for a total of 125 mg/kg, and weekly maintenance doses of 7.5 mg/kg have been used. Although it is safer to administer the drug into the tubing of an intravenous infusion, direct intravenous injections have been given, with the precaution of discarding the needle used to withdraw the drug from the vial in order to avoid subcutaneous reaction. Exemplary doses may be 100 mg/m², 150 mg/m², 175 mg/m², 200 mg/m², 225 mg/m², 250 mg/m², 275 mg/m², 300 mg/m², 350 mg/m², 400 mg/m², 425 mg/m², 450 mg/m², 475 mg/m², 500 mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

Bleomycin

Bleomycin is a mixture of cytotoxic glycopeptide antibiotics isolated from a strain of *Streptomyces verticillus*. It is freely soluble in water.

15

Although the exact mechanism of action of bleomycin is unknown, available evidence would seem to indicate that the main mode of action is the inhibition of DNA synthesis with some evidence of lesser inhibition of RNA and protein synthesis.

20

In mice, high concentrations of bleomycin are found in the skin, lungs, kidneys, peritoneum, and lymphatics. Tumor cells of the skin and lungs have been found to have high concentrations of bleomycin in contrast to the low concentrations found in hematopoietic tissue. The low concentrations of bleomycin found in bone marrow may be related to high levels of bleomycin degradative enzymes found in that tissue.

25

In patients with a creatinine clearance of >35 mL per minute, the serum or plasma terminal elimination half-life of bleomycin is approximately 115 minutes. In patients with a creatinine clearance of <35 mL per minute, the plasma or serum

terminal elimination half-life increases exponentially as the creatinine clearance decreases. In humans, 60% to 70% of an administered dose is recovered in the urine as active bleomycin.

5 Bleomycin should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents in squamous cell carcinoma such as head and neck (including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, larynx), skin, penis,
10 cervix, and vulva. It has also been used in the treatment of lymphomas and testicular carcinoma.

 Because of the possibility of an anaphylactoid reaction, lymphoma patients should be treated with two units or less for the first two doses. If no acute reaction
15 occurs, then the regular dosage schedule may be followed.

 Improvement of Hodgkin's Disease and testicular tumors is prompt and noted within 2 weeks. If no improvement is seen by this time, improvement is unlikely. Squamous cell cancers respond more slowly, sometimes requiring as long as 3 weeks
20 before any improvement is noted.

 Bleomycin may be given by the intramuscular, intravenous, or subcutaneous routes.

25 **Miscellaneous Agents**

Cisplatin

Cisplatin has been widely used to treat cancers such as metastatic testicular or ovarian carcinoma, advanced bladder cancer, head or neck cancer, cervical cancer,

lung cancer or other tumors. Cisplatin can be used alone or in combination with other agents, with efficacious doses used in clinical applications of 15-20 mg/m² for 5 days every three weeks for a total of three courses. Exemplary doses may be 0.50 mg/m², 1.0mg/m², 1.50 mg/m², 1.75 mg/m², 2.0 mg/m², 3.0 mg/m², 4.0 mg/m², 5.0 mg/m², 10mg/m². Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

Cisplatin is not absorbed orally and must therefore be delivered *via* injection intravenously, subcutaneously, intratumorally or intraperitoneally.

10

In certain aspects of the current invention cisplatin is used in combination with emodin or emodin-like compounds in the treatment of non-small cell lung carcinoma. It is clear, however, that the combination of cisplatin and emodin and or emodin-like compounds could be used for the treatment of any other *neu*-mediated cancer.

15

VP16

VP16 is also known as etoposide and is used primarily for treatment of testicular tumors, in combination with bleomycin and cisplatin, and in combination with cisplatin for small-cell carcinoma of the lung. It is also active against non-Hodgkin's lymphomas, acute nonlymphocytic leukemia, carcinoma of the breast, and Kaposi's sarcoma associated with acquired immunodeficiency syndrome (AIDS).

20

VP16 is available as a solution (20 mg/ml) for intravenous administration and as 50-mg, liquid-filled capsules for oral use. For small-cell carcinoma of the lung, the intravenous dose (in combination therapy) is can be as much as 100 mg/m² or as little as 2 mg/m², routinely 35 mg/m², daily for 4 days, to 50 mg/m², daily for 5 days have also been used. When given orally, the dose should be doubled. Hence the doses for small cell lung carcinoma may be as high as 200-250mg/m². The intravenous dose for testicular cancer (in combination therapy) is 50 to 100 mg/m² daily for 5 days, or 100

25

mg/m² on alternate days, for three doses. Cycles of therapy are usually repeated every 3 to 4 weeks. The drug should be administered slowly during a 30- to 60-minute infusion in order to avoid hypotension and bronchospasm, which are probably due to the solvents used in the formulation.

5

Tumor Necrosis Factor

Tumor Necrosis Factor [TNF; Cachectin] is a glycoprotein that kills some kinds of cancer cells, activates cytokine production, activates macrophages and endothelial cells, promotes the production of collagen and collagenases, is an inflammatory mediator and also a mediator of septic shock, and promotes catabolism, fever and sleep. Some infectious agents cause tumor regression through the stimulation of TNF production. TNF can be quite toxic when used alone in effective doses, so that the optimal regimens probably will use it in lower doses in combination with other drugs. Its immunosuppressive actions are potentiated by gamma-interferon, so that the combination potentially is dangerous. A hybrid of TNF and interferon- α also has been found to possess anti-cancer activity.

10
15

Plant Alkaloids

20

Taxol

Taxol is an experimental antimitotic agent, isolated from the bark of the ash tree, *Taxus brevifolia*. It binds to tubulin (at a site distinct from that used by the vinca alkaloids) and promotes the assembly of microtubules. Taxol is currently being evaluated clinically; it has activity against malignant melanoma and carcinoma of the ovary. Maximal doses are 30 mg/m² per day for 5 days or 210 to 250 mg/m² given once every 3 weeks. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

25

Vincristine

Vincristine blocks mitosis and produces metaphase arrest. It seems likely that most of the biological activities of this drug can be explained by its ability to bind specifically to tubulin and to block the ability of protein to polymerize into
5 microtubules. Through disruption of the microtubules of the mitotic apparatus, cell division is arrested in metaphase. The inability to segregate chromosomes correctly during mitosis presumably leads to cell death.

The relatively low toxicity of vincristine for normal marrow cells and
10 epithelial cells make this agent unusual among anti-neoplastic drugs, and it is often included in combination with other myelosuppressive agents.

Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each
15 drug in plasma is approximately 0.4 mM.

Vinblastine and vincristine bind to plasma proteins. They are extensively concentrated in platelets and to a lesser extent in leukocytes and erythrocytes.

20 Vincristine has a multiphasic pattern of clearance from the plasma: the terminal half-life is about 24 hours. The drug is metabolized in the liver, but no biologically active derivatives have been identified. Doses should be reduced in patients with hepatic dysfunction. At least a 50% reduction in dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

25

Vincristine sulfate is available as a solution (1 mg/ml) for intravenous injection. Vincristine used together with corticosteroids is presently the treatment of choice to induce remissions in childhood leukemia: the optimal dosages for these drugs appear to be vincristine, intravenously, 2 mg/m² of body-surface area, weekly,

and prednisone, orally, 40 mg/m², daily. Adult patients with Hodgkin's disease or non-Hodgkin's lymphomas usually receive vincristine as a part of a complex protocol. When used in the MOPP regimen, the recommended dose of vincristine is 1.4 mg/m². High doses of vincristine seem to be tolerated better by children with leukemia than by adults, who may experience severe neurological toxicity. Administration of the drug more frequently than every 7 days or at higher doses seems to increase the toxic manifestations without proportional improvement in the response rate. Precautions should also be used to avoid extravasation during intravenous administration of vincristine. Vincristine (and vinblastine) can be infused into the arterial blood supply of tumors in doses several times larger than those that can be administered intravenously with comparable toxicity.

Vincristine has been effective in Hodgkin's disease and other lymphomas. Although it appears to be somewhat less beneficial than vinblastine when used alone in Hodgkin's disease, when used with mechlorethamine, prednisone, and procarbazine (the so-called MOPP regimen), it is the preferred treatment for the advanced stages (III and IV) of this disease. In non-Hodgkin's lymphomas, vincristine is an important agent, particularly when used with cyclophosphamide, bleomycin, doxorubicin, and prednisone. Vincristine is more useful than vinblastine in lymphocytic leukemia. Beneficial response have been reported in patients with a variety of other neoplasms, particularly Wilms' tumor, neuroblastoma, brain tumors, rhabdomyosarcoma, and carcinomas of the breast, bladder, and the male and female reproductive systems.

Doses of vincristine for use will be determined by the clinician according to the individual patients need. 0.01 to 0.03mg/kg or 0.4 to 1.4mg/m² can be administered or 1.5 to 2mg/m² can also be administered. Alternatively 0.02 mg/m², 0.05 mg/m², 0.06 mg/m², 0.07 mg/m², 0.08 mg/m², 0.1 mg/m², 0.12 mg/m², 0.14 mg/m², 0.15 mg/m², 0.2 mg/m², 0.25mg/m² can be given as a constant intravenous

infusion. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

Vinblastine

5 When cells are incubated with vinblastine, dissolution of the microtubules occurs. Unpredictable absorption has been reported after oral administration of vinblastine or vincristine. At the usual clinical doses the peak concentration of each drug in plasma is approximately 0.4 mM. Vinblastine and vincristine bind to plasma proteins. They are extensively concentrated in platelets and to a lesser extent in
10 leukocytes and erythrocytes.

After intravenous injection, vinblastine has a multiphasic pattern of clearance from the plasma; after distribution, drug disappears from plasma with half-lives of approximately 1 and 20 hours.

15 Vinblastine is metabolized in the liver to biologically activate derivative desacetylvinblastine. Approximately 15% of an administered dose is detected intact in the urine, and about 10% is recovered in the feces after biliary excretion. Doses should be reduced in patients with hepatic dysfunction. At least a 50% reduction in
20 dosage is indicated if the concentration of bilirubin in plasma is greater than 3 mg/dl (about 50 mM).

Vinblastine sulfate is available in preparations for injection. The drug is given intravenously; special precautions must be taken against subcutaneous extravasation,
25 since this may cause painful irritation and ulceration. The drug should not be injected into an extremity with impaired circulation. After a single dose of 0.3 mg/kg of body weight, myelosuppression reaches its maximum in 7 to 10 days. If a moderate level of leukopenia (approximately 3000 cells/mm³) is not attained, the weekly dose may be increased gradually by increments of 0.05 mg/kg of body weight. In regimens

designed to cure testicular cancer, vinblastine is used in doses of 0.3 mg/kg every 3 weeks irrespective of blood cell counts or toxicity.

The most important clinical use of vinblastine is with bleomycin and cisplatin in the curative therapy of metastatic testicular tumors. Beneficial responses have been reported in various lymphomas, particularly Hodgkin's disease, where significant improvement may be noted in 50 to 90% of cases. The effectiveness of vinblastine in a high proportion of lymphomas is not diminished when the disease is refractory to alkylating agents. It is also active in Kaposi's sarcoma, neuroblastoma, and Letterer-Siwe disease (histiocytosis X), as well as in carcinoma of the breast and choriocarcinoma in women.

Doses of vinblastine for use will be determined by the clinician according to the individual patients need. 0.1 to 0.3mg/kg can be administered or 1.5 to 2mg/m² can also be administered. Alternatively, 0.1 mg/m², 0.12 mg/m², 0.14 mg/m², 0.15 mg/m², 0.2 mg/m², 0.25 mg/m², 0.5 mg/m², 1.0 mg/m², 1.2 mg/m², 1.4 mg/m², 1.5 mg/m², 2.0 mg/m², 2.5 mg/m², 5.0 mg/m², 6 mg/m², 8 mg/m², 9 mg/m², 10 mg/m², 20 mg/m², can be given. Of course, all of these dosages are exemplary, and any dosage in-between these points is also expected to be of use in the invention.

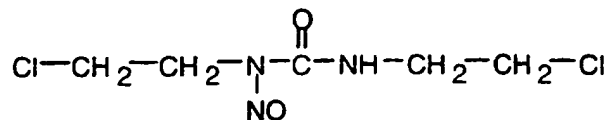
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Alkylating Agents

Carmustine

Carmustine (sterile carmustine) is one of the nitrosoureas used in the treatment of certain neoplastic diseases. It is 1,3bis (2-chloroethyl)-1-nitrosourea. It is lyophilized pale yellow flakes or congealed mass with a molecular weight of 214.06. It is highly soluble in alcohol and lipids, and poorly soluble in water. Carmustine is administered by intravenous infusion after reconstitution as recommended. The structural formula is:

25



5

Sterile carmustine is commonly available in 100 mg single dose vials of lyophilized material.

10 Although it is generally agreed that carmustine alkylates DNA and RNA, it is not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamylation of amino acids in proteins.

15 Carmustine is indicated as palliative therapy as a single agent or in established combination therapy with other approved chemotherapeutic agents in brain tumors such as glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma, and metastatic brain tumors. Also it has been used in combination with prednisone to treat multiple myeloma. Carmustine has proved useful, in the treatment of Hodgkin's Disease and in non-Hodgkin's lymphomas, as secondary therapy in
20 combination with other approved drugs in patients who relapse while being treated with primary therapy, or who fail to respond to primary therapy.

The recommended dose of carmustine as a single agent in previously untreated patients is 150 to 200 mg/m² intravenously every 6 weeks. This may be given as a
25 single dose or divided into daily injections such as 75 to 100 mg/m² on 2 successive days. When carmustine is used in combination with other myelosuppressive drugs or in patients in whom bone marrow reserve is depleted, the doses should be adjusted accordingly. Doses subsequent to the initial dose should be adjusted according to the hematologic response of the patient to the preceding dose. It is of course understood

that other doses may be used in the present invention for example 10mg/m², 20mg/m², 30mg/m², 40mg/m², 50mg/m², 60mg/m², 70mg/m², 80mg/m², 90mg/m², 100mg/m². The skilled artisan is directed to, "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject

Melphalan

Melphalan also known as alkeran, L-phenylalanine mustard, phenylalanine mustard, L-PAM, or L-sarcolysin, is a phenylalanine derivative of nitrogen mustard. Melphalan is a bifunctional alkylating agent which is active against selective human neoplastic diseases. It is known chemically as 4-[bis(2-chloroethyl)amino]-L-phenylalanine.

Melphalan is the active L-isomer of the compound and was first synthesized in 1953 by Bergel and Stock; the D-isomer, known as medphalan, is less active against certain animal tumors, and the dose needed to produce effects on chromosomes is larger than that required with the L-isomer. The racemic (DL-) form is known as merphalan or sarcolysin. Melphalan is insoluble in water and has a pK_{a1} of ~2.1. Melphalan is available in tablet form for oral administration and has been used to treat multiple myeloma.

Available evidence suggests that about one third to one half of the patients with multiple myeloma show a favorable response to oral administration of the drug.

25

Melphalan has been used in the treatment of epithelial ovarian carcinoma. One commonly employed regimen for the treatment of ovarian carcinoma has been to administer melphalan at a dose of 0.2 mg/kg daily for five days as a single course. Courses are repeated every four to five weeks depending upon hematologic tolerance

(Smith and Rutledge, 1975; Young *et al.*, 1978). Alternatively the dose of melphalan used could be as low as 0.05mg/kg/day or as high as 3mg/kg/day or any dose in between these doses or above these doses. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible
5 for administration will, in any event, determine the appropriate dose for the individual subject

Cyclophosphamide

Cyclophosphamide is 2*H*-1,3,2-Oxazaphosphorin-2-amine,
10 *N,N*-bis(2-chloroethyl)tetrahydro-, 2-oxide, monohydrate; termed Cytosan available from Mead Johnson; and Neosar available from Adria. Cyclophosphamide is prepared by condensing 3-amino-1-propanol with *N,N*-bis(2-chloroethyl) phosphoramidic dichloride [(ClCH₂CH₂)₂N--POCl₂] in dioxane solution under the catalytic influence of triethylamine. The condensation is double, involving both the
15 hydroxyl and the amino groups, thus effecting the cyclization.

Unlike other β -chloroethylamino alkylators, it does not cyclize readily to the active ethyleneiminium form until activated by hepatic enzymes. Thus, the substance is stable in the gastrointestinal tract, tolerated well and effective by the oral and
20 parental routes and does not cause local vesication, necrosis, phlebitis or even pain.

Suitable doses for adults include, orally, 1 to 5 mg/kg/day (usually in combination), depending upon gastrointestinal tolerance; or 1 to 2 mg/kg/day; intravenously, initially 40 to 50 mg/kg in divided doses over a period of 2 to 5 days or
25 10 to 15 mg/kg every 7 to 10 days or 3 to 5 mg/kg twice a week or 1.5 to 3 mg/kg/day

A dose 250mg/kg/day may be administered as an antineoplastic. Because of gastrointestinal adverse effects, the intravenous route is preferred for loading. During maintenance, a leukocyte count of 3000 to 4000/mm³ usually is desired. The drug also sometimes is administered intramuscularly, by infiltration or into body cavities.

It is available in dosage forms for injection of 100, 200 and 500 mg, and tablets of 25 and 50 mg the skilled artisan is referred to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 61, incorporate herein as a reference, for details on doses for administration.

5

Chlorambucil

Chlorambucil (also known as leukeran) was first synthesized by Everett *et al.* (1953). It is a bifunctional alkylating agent of the nitrogen mustard type that has been found active against selected human neoplastic diseases. Chlorambucil is known
10 chemically as 4-[bis(2-chlorethyl)amino] benzenebutanoic acid.

Chlorambucil is available in tablet form for oral administration. It is rapidly and completely absorbed from the gastrointestinal tract. After single oral doses of 0.6-1.2 mg/kg, peak plasma chlorambucil levels are reached within one hour and the
15 terminal half-life of the parent drug is estimated at 1.5 hours. 0.1 to 0.2mg/kg/day or 3 to 6mg/m²/day or alternatively 0.4mg/kg may be used for antineoplastic treatment. Treatment regimes are well know to those of skill in the art and can be found in the "Physicians Desk Reference" and in "Remingtons Pharmaceutical Sciences" referenced herein.

20

Chlorambucil is indicated in the treatment of chronic lymphatic (lymphocytic) leukemia, malignant lymphomas including lymphosarcoma, giant follicular lymphoma and Hodgkin's disease. It is not curative in any of these disorders but may produce clinically useful palliation.

25

Busulfan

Busulfan (also known as myleran) is a bifunctional alkylating agent. Busulfan is known chemically as 1,4-butanediol dimethanesulfonate.

Busulfan is not a structural analog of the nitrogen mustards. Busulfan is available in tablet form for oral administration. Each scored tablet contains 2 mg busulfan and the inactive ingredients magnesium stearate and sodium chloride.

5 Busulfan is indicated for the palliative treatment of chronic myelogenous (myeloid, myelocytic, granulocytic) leukemia. Although not curative, busulfan reduces the total granulocyte mass, relieves symptoms of the disease, and improves the clinical state of the patient. Approximately 90% of adults with previously
10 untreated chronic myelogenous leukemia will obtain hematologic remission with regression or stabilization of organomegaly following the use of busulfan. It has been shown to be superior to splenic irradiation with respect to survival times and maintenance of hemoglobin levels, and to be equivalent to irradiation at controlling splenomegaly.

15 ***Lomustine***

Lomustine is one of the nitrosoureas used in the treatment of certain neoplastic diseases. It is 1-(2-chloro-ethyl)-3-cyclohexyl-1 nitrosourea. It is a yellow powder with the empirical formula of $C_9H_{16}ClN_3O_2$ and a molecular weight of 233.71. Lomustine is soluble in 10% ethanol (0.05 mg per mL) and in absolute alcohol (70 mg
20 per mL). Lomustine is relatively insoluble in water (<0.05 mg per mL). It is relatively unionized at a physiological pH. Inactive ingredients in lomustine capsules are: magnesium stearate and mannitol.

Although it is generally agreed that lomustine alkylates DNA and RNA, it is
25 not cross resistant with other alkylators. As with other nitrosoureas, it may also inhibit several key enzymatic processes by carbamoylation of amino acids in proteins.

Lomustine may be given orally. Following oral administration of radioactive lomustine at doses ranging from 30 mg/m² to 100 mg/m², about half of the radioactivity given was excreted in the form of degradation products within 24 hours.

- 5 The serum half-life of the metabolites ranges from 16 hours to 2 days. Tissue levels are comparable to plasma levels at 15 minutes after intravenous administration.

10 Lomustine has been shown to be useful as a single agent in addition to other treatment modalities, or in established combination therapy with other approved chemotherapeutic agents in both primary and metastatic brain tumors, in patients who have already received appropriate surgical and/or radiotherapeutic procedures. It has also proved effective in secondary therapy against Hodgkin's Disease in combination with other approved drugs in patients who relapse while being treated with primary therapy, or who fail to respond to primary therapy.

15

 The recommended dose of lomustine in adults and children as a single agent in previously untreated patients is 130 mg/m² as a single oral dose every 6 weeks. In individuals with compromised bone marrow function, the dose should be reduced to 100 mg/m² every 6 weeks. When lomustine is used in combination with other
20 myelosuppressive drugs, the doses should be adjusted accordingly. It is understood that other doses may be used for example, 20mg/m² 30mg/m², 40 mg/m², 50mg/m², 60mg/m², 70mg/m², 80mg/m², 90mg/m², 100mg/m², 120mg/m² or any doses between these figures as determined by the clinician to be necessary for the individual being treated.

25

C. Pharmaceutical Compositions and Routes of Administration

Aqueous compositions of the present invention will have an effective amount of emodin or emodin-like compound alone or in combination with an effective

amount of a compound (second agent) that is a chemotherapeutic agent as exemplified above. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

5 The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in the therapeutic compositions is contemplated. Supplementary active ingredients, such as other anti-cancer agents, can also be incorporated into the compositions.

15

 In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; time release capsules; and any other form currently used, including cremes, lotions, mouthwashes, inhalents and the like.

20

Parenteral Administration

25 The active compounds of the present invention will often be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, sub-cutaneous, or even intraperitoneal routes. The preparation of an aqueous composition that contains emodin or emodin-like compounds alone or in combination with a second agent as active ingredients will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be

prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

5 Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of
10 microorganisms.

 The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile
15 injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

20 The active compounds may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl
25 groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as
5 lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.
10 Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds
15 in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the
20 preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

25 In certain cases, the therapeutic formulations of the invention could also be prepared in forms suitable for topical administration, such as in cremes and lotions. These forms may be used for treating skin-associated diseases, such as various sarcomas.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, with even drug release capsules and the like being employable.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

D. Kits

All the essential materials and reagents required for inhibiting tumor cell proliferation may be assembled together in a kit. When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred.

For *in vivo* use, emodin or emodin-like compound, alone or in combination with, a chemotherapeutic agent may be formulated into a single or separate pharmaceutically acceptable syringeable composition. In this case, the container

means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the lungs, injected into an animal, or even applied to and mixed with the other components of the kit.

5

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means. The kits of the invention may also include an instruction sheet defining administration of the emodin-like tyrosine kinase inhibitor and/or the chemotherapeutic drug.

10

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, e.g., injection or blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

15

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* * *

EXAMPLE 1

Methodology

25

This example relates to the methods used in the current invention. Those of skill in the art will be able to adapt these methods to the specific requirements of each system

Cell lines and culture. Human breast cancer cell lines MDA-MB453, BT-483, MDA-MB231, MCF-7 and immortalized breast cell line HBL-100 were obtained from the American Type Culture Collection (Rockville, MD). AU-565 cells
5 were obtained from the Naval Bioscience Laboratory (Oakland, CA). MDA-MB453, BT483 and AU-565 cells overexpress *neu* whereas MDA-MB231, MCF-7, and HBL-100 cells express basal level of *neu*.

Human lung cancer cell lines NCI-H226, NCI-H1435, and NCI-H460 were
10 also obtained from the American Type Culture Collection (Rockville, MD). NCI-H1435 cells overexpress *neu*, whereas NCI-H226 and NCI-H460 cells express very low levels of *neu* (Tsai *et al.*, 1993). H460.eB2, H460.eB3 and H460.neo are stable transfectants derived from NCI-460 cells (Yu *et al.*, 1994). H460.eB2 and H460.eB3 express high levels of *neu* expressing vector. H460.neo contains only
15 pSV2-neo drug-selection plasmid and serves in this study as a control (Yu *et al.*, 1994).

All cells were grown in Dulbeccos's modified Eagle's medium/F12 (GIBCO, Grand island, NY) supplemented with 10% fetal bovine serum and gentamicin (50
20 mg/ml). Cells were grown in a humidified incubator at 37°C under 5% CO₂ in air.

Immunoblotting. As described previously (Yu *et al.*, 1990), cells treated overnight in the presence or absence of emodin and in the absence of serum were washed three times with PBS and then lysed in lysis buffer (20 mM Na₂ PO₄, pH 7.4;
25 150 mM NaCl; 1% Triton X-100; 1% aprotinin; 1 mM phenylmethysulfonyl fluoride; 10 mg/ml leupeptin; 100 mM NaF and 2 mM Na₃VO₄). Protein contents were determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 500 mg of protein was separated by 6% SDS-PAGE and transferred to nitrocellulose filter paper (Schleicher & Schuell.

Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing non-fat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). The treated filter paper was incubated with primary antibodies (the anti-p185^{neu} antibody *c-neu* [Ab-3] for detection of p185^{neu} or the anti-phosphotyrosine antibody [UBI, Lake Placid, NY] for detection of phosphotyrosine), and incubated with HRP-goat anti-mouse antibody (1:1000 dilution) (Boehringer Mannheim Corp., Indianapolis, IN). Bands were visualized with the enhanced Chemiluminescence system (Amersham Corp., Arlington Heights, IL).

10

Immuno-complex Kinase Assay. The immuno-complex kinase assay was modified from those described previously (Kiyokawa *et al.*, 1995). Briefly, cells were treated with or without 40 μ M emodin for 24 h, then washed 3 times with PBS. Cells were then collected and lysed in lysis buffer. Cell lysates (500 mg) were incubated with monoclonal anti-p185^{neu} antibody *c-neu* (Ab-3) for 1 h at 4°C, then precipitated with 50 ml of protein-A-conjugated agarose (Boehringer Mannheim) for 30 minutes at 4°C, and washed 3 times with 50 mM Tris-HCl buffer containing 0.5 M LiCl (pH 7.5) and once in assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM MnCl₂). To 40 ml of beads (protein-a-conjugated agarose), 10 μ Ci of [γ -³²P]ATP (Amersham) and 10 ml of enolase (Sigma Chemical Co., St. Louis, MO) were added and incubated for 20 minutes at room temperature. The reactants were separated by 7.5% SDS-PAGE. The gel was dried and visualized by autoradiography.

15

20

Proliferation assay. Cells were detached by trypsinization, seeded at 2×10^4 cells/ml in a 96-well microtiter plate overnight, then treated with various concentrations of test samples and incubated for an additional 72 h. The effects on cell growth of emodin, cisplatin, doxorubicin, or VP16 alone or in combination were examined by MTT assay (Mosmann *et al.*, 1983; Rubinstein *et al.*, 1990). Briefly, 20

25

5 μl of MTT solution (5 mg/ml) (Sigma Chemical Co., St. Louis, MO) was added to each well and incubated for 4 h at 37°C. The supernatant was aspirated, and the MTT formazan formed by metabolical viable cells was dissolved in 150 μl of dimethyl sulfoxide, then monitored by a microplate reader (Dynatech MR 5000 fluorescence, Dynatech Corp., Burlington, MA) at a wavelength of 590 nm.

10 **Colony Formation in Soft Agarose.** As described previously (Yu *et al.*, 1993), cells (1×10^3 cells/ml) were seeded in 24-well plates in culture medium containing 0.35% agarose (FMC Corp., Rockland, ME) over a 0.7% agarose layer and incubated for 3 weeks at 37°C. Colonies were then stained with p-iodonitrotetrazolium violet (1mg/ml), and colonies larger than 100μm were counted. Each determination was performed 4 times.

15 **Lipid Visualization.** As described previously (Sheehan, 1980) a modified Oil Red O in propylene glycol method was used to visualize neutral lipids (Bacus *et al.*, 1992; Bacus *et al.*, 1990).

20 **Evaluation of drug combination.** For evaluating the combined effect of the two drugs, observed values were compared to predicted values (c) calculated from the equation $c = a \times b / 100$, where a and b indicate survival values with single agents (Webb, 1963; Hata *et al.*, 1994). Observed values of less than 70% of predicted ones were considered synergistic.

25 **Zymography of Gelatinolytic Activity.** As described previously (Yu and Hung, 1991). Cells were detached by trypsinization, seeded at 2×10^6 cells/well in a 6-well plate and cultured in DMEM/F12 medium supplemented with 1% FBS for overnight, washed cells with PBS, added serum-free DMEM/F12 medium, then treated with various concentrations of test samples and incubated for an additional 24

h. The culture supernatants were collected, centrifuged at $800 \times g$ for 10 min. then at $18,000 \times g$ for 10 min. The supernatants (150 μ l) were analyzed by zymography using SDS-PAGE containing 1.5% gelatin prepared according to procedures described previously (Yu and Hung, 1991).

5

In Vitro Chemoinvasion Assay. In vitro invasiveness was carried out according to the procedure described previously (Yusa *et al.*, 1990; 1994) with modifications. Briefly, 24 well Transwell unites with an 8- μ m pore size polycarbonate filter (Costar Corp., Cambridge, MA) were coated with 0.1 ml of a 1:30 dilution (48 μ g/filter) of Matrigel (Yusa *et al.*, 1990; Yu *et al.*, 1994) in cold DMEM/F12 medium. then air dried these filters at room temperature, and formed a thin continuous layer on top of the filter. The lower compartment contained 0.6 ml laminin (20 mg/ml, Becton Dickinson) as chemoattractant or DMEM/F12 medium as a negative control. The cells (1×10^5 cells/0.1 ml of DMEM/F12 containing 0.1% bovine serum albumin) were placed in the upper compartment and incubated with or without either emodin or DK-V-47 for 72 h at 37 $^{\circ}$ C in a humidified 95% air, 5% CO₂. After the incubation, the filters were fixed with 3% glutaraldehyde in PBS and stained with Giemsa, then counted the number of cells per High-power field (X 200) that had migrated to the lower side of the filter.

20

EXAMPLE 2

Effect of Emodin on Tyrosine Phosphorylation in Breast Cancer Cells that Overexpress *neu*

25 To test whether emodin, a tyrosine kinase inhibitor for the protein tyrosine kinase p56^{lck} (Jayasuriya *et al.*, 1992), may also inhibit *neu* tyrosine kinase, human breast cancer cells MDA-MB453 that overexpress p185^{neu} were used to test the effect of emodin on tyrosine phosphorylation of p185^{neu}.

Cells were treated with different concentrations of emodin at 37°C for 24 h, then analyzed for the protein level of p185^{neu} and its tyrosine phosphorylation. The p185^{neu} was first immunoprecipitated by anti-p185^{neu} antibody and the immunoprecipitates were then blotted with anti-phosphotyrosine antibody for detection of phosphotyrosine or anti-p185^{neu} antibody for p185^{neu} detection.

Emodin, at a 40 µM concentration, induced a significant reduction in the level of tyrosine phosphorylation (FIG. 1A), but had no effect on p185^{neu} protein level. The reduced tyrosine phosphorylation of p185^{neu} could be readily detected after 12 h (FIG. 1B).

To further confirm that the reduced tyrosine phosphorylation by emodin is a general phenomenon for p185^{neu}, other *neu*-overexpressing breast cancer cell lines were also examined and similar results were obtained (FIG. 2). These cell lines include BT-483, AU-565 (FIG. 2), SKBr-3, and MDA-MB361.

When MCF-7 cells that express basal level of p185^{neu} protein were examined similarly, phosphotyrosine level of p185^{neu} was almost undetectable under the experimental condition. This makes it insignificant to compare the effect of emodin on phosphotyrosine level on p185^{neu} in MCF-7 cells.

To test relationship between chemical structure and inhibitory activity of emodin and its derivatives on tyrosine phosphorylation of *HER-2/neu* and proliferation of *HER-2/neu*-overexpressing breast cancer cells, 12 derivatives of emodin as shown in Table 1, were synthesized and according to substituent at the different structure position of emodin, these derivatives were separated into five groups. Human breast cancer MDA-MB 453 cells which overexpress p185^{neu} were

treated with varying concentrations of emodin and 12 derivatives at 37°C for 24 hr, then analyzed for the protein level of p185^{neu} and its tyrosine phosphorylation using immunoblotting with anti-phosphotyrosine antibody for detection of phosphotyrosine (anti-PY) or anti-p185^{neu} antibody for detection of p185^{neu}.

5

As show in the Table 2. in all compounds tested, emodin's carbon 9 (C9) group substitute. named DK-V-47 is the most effective to suppress tyrosine phosphorylation of p185^{neu}, the parental compound emodin is less effective than DK-V-47. The concentration of emodin and DK-V-47 needed for 50% inhibitory activity of tyrosine phosphorylation are 21 µM. and 17 µM. respectively. Under the same condition. emodin and its derivatives did not affect protein levels of p185^{neu}. A number of other inhibitors listed herein below also had a marked inhibitory on proliferation of cells even though their

10

Table 2. Relationship between chemical structure of emodin and its derivatives and inhibitory activity on tyrosine phosphorylation of *neu* and proliferation of MDA-MB 453 cells. Cells in the serum-free medium were incubated with emodin or its derivatives (0, 10, 20, 30, 40, 60, 80 and 100 μ M) at 37°C for 24 h, then blotted with antiphosphotyrosine antibody and quantified with density scanner program; (b), cells were treated with different concentrations of emodin and derivatives for 72 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment of emodin and derivatives as 100%. All determinations were made in triplicate. Results are mean \pm SD.

| Compound | Inhibitory Activity Of Emodin And Derivatives On | |
|--|--|------------------------|
| | Tyrosine phosphorylation IC50 (μ M) | Proliferation of cells |
| Group A. C₃ Groups: | | |
| Emodin | 21 | 5 |
| DK-III-8 | >100 | 81 |
| DK-III-19 | >100 | 45 |
| DK-III-47 | 39 | 50 |
| DK-III-48 | 67 | 35 |
| DK-III-13 | 100 | 60 |
| DK-III-11 | >100 | >100 |
| Group B. C₆ Groups: | | |
| DK-II-1 | >100 | >100 |
| DK-II-2 | >100 | 100 |
| Group C. C₁, C₂ Groups: | | |
| DK-IV-1 | >100 | >100 |
| Group D. C₉ Groups: | | |
| DK-V-47 | 17 | 1 |
| DK-V-48 | >100 | >100 |
| Group E. C₁₀ Group: | | |
| DK-III-52 | >100 | >100 |

The results presented elsewhere in this specification show that emodin effectively suppresses tyrosine kinase activity of *HER-2/neu*, and inhibits growth of *HER-2/neu*-overexpressing human breast cancer cells (see also Zhang *et al.*, 1995). The inventors also examined the effect of emodin and its derivatives on proliferation of MDA-MB 453 cells. Cells were treated with or without different concentrations of emodin and 12 derivatives at 37°C for 72 hr, then tested using MTT assay. As shown in Table 2, in the all compounds examined, DK-V-47 is the most effective to against these cell growth. Emodin has 5-fold less activity than DK-V-47 to inhibit cell proliferation. Suppression of Emodin and its derivatives on tyrosine phosphorylation of p185^{neu} correlates with the effect these compounds' effect on inhibition of cell proliferation (Table 2).

These results suggest that CH₃ group at C₃ position, and OH group at C₁, C₆, and C₈ position are very important for maintaining inhibitory activity of emodin to suppress tyrosine phosphorylation of *HER-2/neu*, and to block growth of *HER-2/neu*-overexpressing breast cancer cells. However, oxygen atom of ketone at C₁₀ is replaced by p-ethyl nitro-benzene-methyl-group (DK-V-47) show more effective than emodin for both inhibitory activity of tyrosine phosphorylation of p185^{neu} and proliferation of *HER-2/neu*-overexpressing breast cancer cells. Interestingly, when ethyl nitro group of DK-V-47 is substituted by nitro group (DK-V-48), the compound show a little activity, compare with DK-V-47. These results suggest that COCH₃ group of DK-V-47 plays a important role in maintaining DK-V-47 inhibitory activity for tyrosine phosphorylation of p185^{neu} and proliferation of breast cancer cells.

EXAMPLE 3

Repression of Autophosphorylation and Transphosphorylation by Emodin *in vitro*

- 5 The results from Example 2 show that phosphotyrosine level of p185^{neu} is repressed by emodin. To examine whether this reduction in tyrosine phosphorylation affects the tyrosine kinase activity of p185^{neu}, the immuno-complex kinase assay was used.

10 When MDA-MB453 cells were treated with emodin for 24 h. the autophosphorylation ability of the p185^{neu} from these cells was inhibited. Further, the transphosphorylation ability of p185^{neu} for enolase, an exogenous substrate for tyrosine kinase, was also significantly decreased compared with untreated cells (FIG. 3A). Hence, emodin-treatment of cells results in reduced phosphotyrosine levels in p185^{neu}, which in turn exhibits lower tyrosine kinase activity (FIG. 1 and FIG. 3A).

15

To further address whether emodin inhibits intrinsic tyrosine kinase activity of p185^{neu}, p185^{neu} was immuno-precipitated from the untreated MDA-MB453 cells. The precipitates were treated with different concentrations of emodin and the kinase activity measured. The tyrosine kinase activity for both auto- and trans-phosphorylation of p185^{neu} is inhibited by emodin in a dose-dependence manner (FIG. 3B). These results show conclusively that emodin represses the intrinsic tyrosine kinase activity of p185^{neu}. Therefore, the reduced phosphotyrosine level in p185^{neu} by emodin treatment is most likely caused by inhibition of p185^{neu} tyrosine kinase activity.

20
25

Since activated *HER-2/neu* transformed 3T3 cells possess high tyrosine kinase of p185^{neu} (Stern *et al.*, 1986; Bargmann and Weinberg, 1988), to address whether emodin and DK-V-47 can directly inhibit tyrosine kinase activity of activated

HER-2/neu. p185^{neu} was immunoprecipitated from the untreated activated *HER-2/neu* transformed 3T3 cells. the precipitates were then treated with varying concentrations of emodin and DK-V-47, and the kinase activity was measured. The tyrosine kinase activity for both auto-phosphorylation ability for p185^{neu} and trans-phosphorylation ability for enolase is inhibited by emodin and DK-V-47 in a dose-dependent manner (FIG. 12). Compare with emodin, DK-V-47 is more effective than emodin (FIG. 12). These results conclude that emodin and DK-V-47 inhibit tyrosine kinase activity of activated p185^{neu}. Therefore, the repressed transformation of activated *HER-2/neu* transformed 3T3 is most likely caused by inhibition of p185^{neu} tyrosine kinase activity.

EXAMPLE 4

Effect of Emodin on the Proliferation of Human Breast Cancer Cells

Since emodin effectively inhibits tyrosine kinase activity of p185^{neu} that is critical for cell growth, it is necessary to investigate whether emodin inhibits cell proliferation for the breast cancer cells that overexpress p185^{neu}. To address this issue, six cell lines were chosen for further study.

The MDA-MB453, BT-483 and AU-565 cells are *neu*-overexpressing breast cancer cell lines as mentioned earlier, the MCF-7 and MDA-MB231 are two human breast cancer cell lines expressing basal level of p185^{neu}. The HBL-100 cell line is derived from a normal human breast tissue transformed by SV40 large T antigen and expresses basal level of p185^{neu}. As shown in FIG. 4, growth of these cells was inhibited by emodin in a dose-dependent manner, but to different extent. At 40 μ M concentration that effectively inhibited tyrosine kinase activity of p185^{neu} (FIG. 1, FIG. 2 and FIG. 3), emodin blocked 68%, 72%, and 84% of growth in MDA-MB453,

BT-483 and AU-565 cells, respectively. However, under the same conditions, it only inhibited 37% and 23% of growth in MCF-7 and MDA-MB 231 cells, respectively.

5 Emodin had little effect on HBL-100 breast cells even up to 80 μ M concentration. The differential growth effect was not apparent when the cells were treated with 10 μ M emodin. This concentration of emodin did not affect tyrosine kinase activity of p185^{neu}. These results show that emodin preferentially suppresses growth of the *neu*-overexpressing breast cancer cells and suggests that the differential suppression effect is likely through repression of p185^{neu} tyrosine kinase.

10 Because emodin at 40 μ M concentration, inhibited p185^{neu} tyrosine kinase activity (FIG. 1, FIG. 2 and FIG. 3) and significantly suppressed growth of breast cancer cells that overexpress p185^{neu} (FIG. 4A), the effect of time course on proliferation of MDA-MB453 cells by emodin at 40 μ M concentration was measured.

15 Emodin completely inhibited cancer cell growth, while viability of cells was measured by trypan blue assay, more than 90% of cells were found to be alive (FIG. 4B). The results show that the mechanism that causes inhibition of cell growth is primarily due to repression of proliferation and not induction of cell death under this condition.

20 In a further study the emodin and DK-V-47 effects on cell proliferation preferentially for the activated *HER-2/neu* transformed 3T3 cells was tested. NIH 3T3 parental cell line and its activated *HER-2/neu* transformed cells were used. As shown in FIG. 13, the growth of activated *HER-2/neu* transformed 3T3 cells was inhibited by emodin and DK-V-47 in a dose-dependent manner, but to varying extents. At 80 μ M concentration, emodin and DK-V-47 block 55%, and 83% of growth of activated

HER-2/neu, respectively. However, under the same condition, both emodin and DK-V-47 had little effect on parental 3T3 cells. These results indicate that emodin and DK-V-47 preferentially suppress growth of activated *HER-2/neu* transformed cells and suggested that the differential suppression effect occurs through inhibition of p185^{neu} tyrosine kinase activity.

EXAMPLE 5

Emodin Induces Differentiation of Breast Cancer Cells

As emodin inhibited breast cancer cell growth and no significant cell death were observed (FIG. 4B), it is of interest to investigate whether emodin induces differentiation of breast cancer cells. When MDA-MB453 cells are treated with emodin (40 μ M) for 24 h, cells display a flat morphology with larger nuclei and increased cytoplasm that are characteristic for differentiation (Plowman *et al.*, 1993), compared with untreated cells, which are moderately adherent, with a rounded morphology.

Maturation of breast cells is characterized by the presence of lipid droplets that are milk components. Large droplets containing neutral lipid are readily detectable in emodin-treated cells, in contrast, no large lipid droplets are observed in the untreated cells. More than 90% of the emodin-treated cells produce large lipid droplets, but only 2-5% untreated cells contain lipid drops and these are much smaller in size. These results demonstrate that emodin preferentially induces differentiation of *neu*-overexpressing breast cancer cells, suggesting that the enhanced tyrosine kinase activity of p185^{neu} may prevent breast cancer cells from differentiation.

EXAMPLE 6**Effect of Emodin on Transformation of Breast Cancer Cells**

One hallmark of the transformed state is the ability of cells to exhibit
5 anchorage-independent growth. To determine whether emodin affected this property
in breast cancer cells, cells are seeded into soft agarose and monitored for colony
formation (FIG. 5). The colony formation activity of *neu*-overexpressing breast
cancer cells, MDA-MB453, BT483 and AU565 in soft agarose containing 40 μ M
emodin is dramatically suppressed. However, under the same condition, the cells, that
10 express basal level of p185^{neu}, namely MCF-7, MDA-MB231 and HBL-100 still form
significant number of colonies. The decreased ability to grow in soft agarose for the
neu-overexpressing breast cancer cells does not simply reflect slower proliferation
rate shown in FIG. 4, because additional 3 weeks longer incubation does not increase
number of colonies. Furthermore, no significant change in colony formation activity
15 is observed when the cells grow in soft agarose containing 10 μ M emodin, a
concentration that does not inhibit tyrosine kinase activity of p185^{neu} (FIG. 1 and FIG.
3B).

Taken together, the results indicate that emodin preferentially suppresses the
20 transformation ability of *neu*-overexpressing cancer cells and suggest that the
transformation suppression by emodin may be mediated through its ability to inhibit
tyrosine kinase activity of p185^{neu}.

Whether cells have the ability to exhibit anchorage-independent growth is one
hallmark to detect cell transformed status. Since emodin can repress human breast
25 cancer colonies formation in soft agarose (see above and Zhang *et al.*, 1995) the
inventors decided to test whether DK-V-47 may also affect this property in breast
cancer, cells were seeded into soft agarose, and monitored for colony formation. As
shown in the FIG. 11A, the colony forming activity of *HER-2/neu*-overexpressing

breast cancer MDA-MB-453 cells in soft agarose containing different concentrations of DK-V-47 or emodin was repressed in a dose-dependent manner. However, under the same condition, the MCF-7 cells that express basal levels of p185^{neu}, still formed a significant number of colonies. Interestingly, although DK-V-47 show slightly more effective than emodin to inhibit tyrosine phosphorylation of p185^{neu} and proliferation of MDA-MB 453 cells (Table 2), DK-V-47 show much more effective than emodin to repress colony formation ability of these cells in soft agarose (FIG. 11A). Since activated *HER-2/neu* transformed 3T3 cells have high tyrosine kinase activity and have ability to grow in soft agarose (Stern *et al.*, 1986; Bargmann and Weinberg, 1988). To address whether emodin and DK-V-47 can repress colony formation of activated *HER-2/neu* transformed 3T3 cells, activated transformed 3T3 cells were seeded into soft agarose containing different concentration of emodin and DK-V-47. The colony formation of these cells was then examined. Emodin and DK-V-47 repress colony formation in dose-dependent manner. DK-V-47 is much more effective than emodin (FIG. 11B). These results show that DK-V-47 as well as emodin inhibits colony formation of activated *HER-2/neu* transformed 3T3 cells which may be mediated through suppression of tyrosine kinase activity of p185^{neu} in 3T3 cells.

EXAMPLE 7

Effects of Emodin on Tyrosine Phosphorylation and Cell Proliferation in Human Lung Cancer Cells

This example relates to the *neu* tyrosine kinase inhibitory activity of emodin in lung cancer cells. Emodin inhibits *neu* tyrosine kinase in breast cancer cells as seen in Examples 2 through 6 above. To test whether it also inhibits *neu* tyrosine kinase in human lung cancer cells, the inventors examined the effect of emodin on tyrosine phosphorylation of p185^{neu} in NCI-H1435 and NCI-H226 cells.

Cells are treated with emodin at 37°C for 24 h in the absence of serum, then analyzed for the levels of p185^{neu} and its tyrosine phosphorylation. Emodin, at a concentration of 30 µM, significantly reduces the level of tyrosine phosphorylation in NCI-H1435 cells (FIG. 6A) but has no effect on p185^{neu} level in these cells.

5

The inventors have also examined NCI-H460 and its transfectants (Yu *et al.*, 1994). As expected, the parental NCI-H460 and H460.neo control cells express very low levels of p185^{neu}. The other two transfectants express higher levels of p185^{neu}, their tyrosine phosphorylation of p185^{neu} was inhibited by emodin. These results show that emodin inhibits tyrosine phosphorylation of p185^{neu} in human lung cancer cells.

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EXAMPLE 8

Emodin Preferentially Inhibits Proliferation of neu-overexpressing Human Lung Cancer Cells

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Emodin inhibits the tyrosine kinase activity of p185^{neu} which is critical for cell growth and preferentially inhibits proliferation neu-overexpressing breast cancer cells (Examples 2-6 above). To test whether emodin has similar anti-proliferative activity in lung cancer cells, NCI-H1435, NCI-H226, and NCI-H460 cells and NCI-H460 transfectants are treated with different concentrations of emodin for 3 days.

20

As shown in FIG. 7A and FIG. 7B, growth of NCI-H1435, NCI-H226, and NCI-H460 cells and NCI-H460 cells is inhibited by emodin in a dose-dependent manner, but to different extent. At the 30 µM concentration that effectively inhibits tyrosine phosphorylation of p185^{neu} (FIG. 6), emodin blocks 67% of the growth of NCI-H1435 cells (FIG. 7A). However, under the same conditions, it inhibits only 25% of the growth of NCI-H226 cells (FIG. 7A). Similar results are obtained in

25

NCI-H460 and its transfectants. Emodin inhibited 48% and 52% of the growth of H470.eB2 and H460.eB3, but blocks only 30% and 43% of the growth of parental NCI-H460 cells and H460.neo control cells, respectively (FIG. 7B).

5 These results show that emodin preferentially suppresses growth of the *neu*-overexpressing lung cancer cells and this differential suppression effect is mediated through repression of p185^{neu} tyrosine phosphorylation.

EXAMPLE 9

10 Emodin Sensitized *neu*-Overexpressing Lung Cancer Cells to Cisplatin, Doxorubicin, and VP16

Since emodin suppresses tyrosine phosphorylation of *neu* (FIG. 6A and FIG. 6B), and overexpression *neu* induces resistance to chemotherapeutic drugs (Tsai *et al.*,
15 1993; Tsai *et al.*, 1995), it is imperative to investigate whether emodin sensitizes *neu*-overexpressing lung cancer cells to chemotherapeutic drugs. To explore this possibility, the inventors examined the combined effects of emodin with chemotherapeutic agents on proliferation of NCI-H1435, NCI-H226, and NCI-H460 transfectants.

20 To identify optimal conditions for these combined treatments, the sensitivity of these cells to the chemotherapeutic drugs cisplatin, doxorubicin, and VP16 was determined. The *neu*-overexpressing NCI-H1435 cells show 43-fold more chemoresistance to cisplatin (FIG. 8A), 8.6-fold more resistance to doxorubicin (FIG.
25 8B) and 8.5-fold more resistance to VP16 (FIG. 8C) than NCI-H226 cells that express low levels of *neu*. The *neu*-mediated chemoresistance is also evident in the NCI-H460 transfectants but not in the parental NCI-H460 cells, or the control H460.neo cells. The H460.eB transfectants show chemoresistance to the examined

drugs ranging from 12-fold to 15-fold (FIG. 8A, FIG. 8B and FIG. 8C). These results are consistent with the observation that *neu* expression induces drug resistance.

EXAMPLE 10

5 **The Combined Effect of Emodin and Chemotherapeutic Agents on
Proliferation of NCI-H1435, NCI-H226, and NCI-H460 Cells
and NCI-H460 Transfectants**

10 The growth of NCI-H1435 cells treated with emodin, cisplatin, doxorubicin, or
VP16 alone, is inhibited by only 38%, 19%, 24% and 22%, respectively. The
combinations of emodin with cisplatin, doxorubicin, or VP16 increases the cell
growth inhibitory activity of these chemotherapeutics to 87%, 92% and 100%,
respectively (FIG. 9A). However, no such synergistic effect is observed in NCI-H226
15 cells which express low levels of *neu* (FIG. 9B). Synergistic effects are also observed
in the *neu*-overexpressing H460.eB2 and H460.eB.3 cells (FIG. 9E and FIG. 9F). In
the parental NCI-H460 and control H460.neo cells, however, no significant synergistic
anti-proliferative effect is seen (FIG. 9C and FIG. 9D).

20 These results show that emodin induces synergistic anti-proliferative activity
of cisplatin, doxorubicin, and VP16 on *neu*-overexpressing lung cancer cells.

EXAMPLE 11

25 **Effect of Cisplatin, Doxorubicin and VP16 alone or in combination with
Emodin on human lung cancer cell colony growth in soft agar**

The growth of NCI-H460 cells treated with 30 μ M emodin (E), 5 μ M cisplatin
(Cis), 0.1 μ M doxorubicin (DO), or 0.1 μ M VP16 alone, is inhibited by only 32%, 40,
30% and 18%, respectively. The combinations of emodin with cisplatin, doxorubicin,

or VP16 increases the cell growth inhibitory activity of these chemotherapeutics to 70%, 40%, and 52%, respectively (FIG. 10A). Similarly, the growth of H460.neo cells was inhibited 50% with 30 μ M emodin alone, 25% with 0.1 μ M doxorubicin alone, 48% with 5 μ M cisplatin alone and 0% with 0.1 μ M VP16. However, the combination of emodin with cisplatin, doxorubicin and VP16 increases the growth inhibitory activity of these chemotherapeutics to 72%, 80% and 63%, respectively (FIG. 10B). The growth of H460.eB2 cells was inhibited 55% with 30 μ M emodin alone, 48% with 0.5 μ M doxorubicin alone, 70% with 75 μ M cisplatin alone and 58% with 0.5 μ M VP16. However, the combination of emodin with cisplatin, doxorubicin and VP16 increases the growth inhibitory activity of these chemotherapeutics to above 95% in each case (FIG. 10C). Similarly with H460.eB3 30 μ M emodin inhibited growth by 52%, 0.5 μ M VP16 inhibited growth by 54% 0.5 μ M doxorubicin inhibited growth by 60% and 75 μ M cisplatin inhibited growth by 65%. However, in combination with emodin the inhibitory effects of these chemotherapeutic drugs increases to over 95% in each case (FIG. 10D). The growth of H226 is inhibited by 30% with 30 μ M emodin, 30% with 5 μ M cisplatin, 25% with 0.1 μ M doxorubicin and 28% with 0.1 μ M VP16. However, in combination with emodin the inhibitory activity of these chemotherapeutic drugs increases to 55%, 45% and 50%, respectively (FIG. 10E). The growth of H1435 is inhibited by 40% with 30 μ M emodin, 25% with 50 μ M cisplatin, 35% with 1 μ M doxorubicin and 32% with 1 μ M VP16. However, in combination with emodin the inhibitory activity of these chemotherapeutic drugs increases to above 95% in each case (FIG. 10F).

The results above demonstrated that emodin inhibited tyrosine phosphorylation of *HER-2/neu* in *HER-2/neu* transfected human lung cancer cells and sensitized the inhibitory effect of chemotherapeutic agents (cisplatin, doxorubicin, and VP16) on the proliferation of *HER-2/neu*-overexpressing lung cancer cells (Zhang and

Hung, 1996). Similar results were also obtained from human breast cancer cells (Fig. 16A and FIG. 16B), Colony formation ability of *HER-2/neu*-overexpressing cancer cells MDA-MB 361 (FIG. 16A) in soft agarose containing either emodin, or taxol alone was suppressed by 28% and 31%, respectively. The combinations of emodin with taxol synergistically increased their inhibitory activity to 96% (Fig. 16A). No such synergistic effect was observed in MDA-MB 435 cells which express low levels of *HER-2/neu* (Fig. 16B). These results indicate that emodin induces synergistic inhibitory activity of taxol on *HER-2/neu*-overexpressing breast cancer cells

10 These results suggested that the tyrosine kinase activity of *HER-2/neu* is required for the chemoresistance of *HER-2/neu*-overexpressing cancer cells: and that addition of emodin may improve the efficacy of chemotherapeutic regimens.

15 *HER-2/neu*-overexpressing human breast cancer cells can form tumors in athymic BALB/c nude mice. To test whether emodin suppresses tumor development in mice bearing *HER-2/neu*-overexpressing cancer cells, tumors were induced by injecting *HER-2/neu*-overexpressing human breast cancer MDA-MB 361 cells (5×10^7 cells/0.1 ml/mouse) subcutaneously (s.c.). Then 3 weeks later, when the solid tumor became palpable, the mice were treated with either emodin [40 mg/kg of body weight] or taxol (10 mg/kg of body weight), or emodin plus taxol [0.2 ml/mouse, intraperitoneal (i.p.) injection] on a 3-days-a-week schedule for 8 weeks. Then mice were observed for survival up to 10 months. Mice treated with placebo (cremophor : DMSO : saline, 1 : 2 : 7) continued to develop tumors and eventually died of tumors between 2-5 months. Either emodin or taxol alone inhibited

20 *HER-2/neu*-overexpressing human breast cancer tumor growth in the nude mice (Fig. 17A), and significantly prolonged the life span of MDA-MB 361 tumor-bearing animals (Fig. 17B). However, mice treated with emodin plus taxol, tumor was gradually shrink, finally 2 out of 5 mice are tumor free (Fig. 17A & 17B). Either

25

emodin alone or emodin plus taxol did not induce mice to lose weight compared with control mice. These results indicated that emodin synergistically suppressed growth of *HER-2/neu*-overexpressing human breast tumor cells, prolonged the life span of *HER-2/neu*-overexpressing tumor mice and suggested that tyrosine kinase inhibitor
5 emodin may be used as a therapeutic agent for sensitizing *HER-2/neu*-overexpressing breast cancers to other *HER-2/neu* resistance drugs, such as taxol.

To investigate whether the therapeutic effects on tumors *in vivo* were due to phosphorylation of *HER-2/neu* tyrosine kinase was suppressed by emodin, tyrosine
10 phosphorylation of *HER-2/neu* in tumors from one mouse in each group were analyzed by immunohistochemical staining using antibody against tyrosine phosphorylation or p185^{*neu*} and Western blot. Tyrosine phosphorylation levels in the emodin treated tumor was almost abolished, compared with control tumor however p185^{*neu*} protein levels in the emodin treated tumor was not significantly changed.
15 These results indicated that emodin suppresses growth of *HER-2/neu* overexpressing tumor in nude mice through inhibiting phosphorylation of *HER-2/neu* tyrosine kinase.

EXAMPLE 12

Emodin and DK-V-47 repress metastasis-associated properties of
20 activated *HER-2/neu* transformed cells.

Tumor metastasis is a complex process involving a sequential series of critical steps (Liotta, 1986; Nicolson, 1988; 1991). During blood-born metastasis, important steps are the impartation of tumor cells in the microcirculation and the subsequent
25 invasion of blood vessel basement membrane. Gelatinase (type IV collagenases) has been shown to be important participant in the dissolution of the basement membrane collagen during tumor cell invasion and metastasis (Jayasuriya *et al.*, 1992). The inventors previous studies demonstrated that activated *HER-2/neu* transformed 3T3

cells can induce metastasis in nude mice and have high gelatinase activities (Yu and Hung, 1991). To test whether emodin and DK-V-47 can decrease the secreted protease (gelatinase collagenase IV) in activated *HER-2/neu* cells, the effect of emodin and DK-V-47 on basement membrane-degradation gelatinase in activated *HER-2/neu* transformed 3T3 cells was examined with zymographic analysis. As shown in FIG. 14A and FIG. 14B, both emodin and DK-V-47 inhibit gelatinolytic activity of the 92 kDa and 68 kDa gelatinase. The inhibition is enhanced with increasing concentration of either DK-V-47 (FIG. 14B) or emodin (FIG. 14A). DK-V-47, at 10 μ M concentration shows significant inhibitory activity for gelatinase. however, emodin, at 40 μ M has the similar effete with DK-V-47 on gelatinase activity. These results show that DK-V-47 is more effective than emodin on inhibition of gelatinase secretion.

Blood-borne malignant cells must extravasate from the circulation. invade basement membrane and colonize distant sites to be metastatic. Therefore, cancer cells invasion is a very important in the metastasis events. Activated *HER-2/neu* transformed 3T3 cells have been shown to be invasive (Yu and Hung, 1991). To examine whether emodin and DK-V-47 can abolish the invasive properties of activated *HER-2/neu* transformed cells, in vitro invasion assays were performed to monitor the effect of emodin and DK-V-47. As shown in the FIG. 15. emodin and DK-V-47 can almost abolish ability of activated *HER-2/neu* transformed cells to penetrate the Matrigel layer, compare with untreated cells (FIG. 15 positive control). However, at the 25 μ M concentration, DK-V-47 is more effective than emodin to repress invasive ability of activated *HER-2/neu* transformed cells. These results demonstrate that emodin and DK-V-47 can completely inhibit the invasive ability of activated *HER-2/neu* transformed 3T3 cells in vitro. The results that emodin and DK-V-47 significantly decreased the 68 kDa and 92 kDa gelatinase production (FIG. 14), and almost abolished ability of *HER-2/neu*-transformed 3T3 cells to invade the

Matrigel (FIG. 15), suggest that emodin and DK-V-47 may suppress metastasis through repressing of p185^{neu} tyrosine kinase activity.

EXAMPLE 13

5 ***In Vivo* Prevention of Breast, Lung and Ovarian Tumor Development in vivo by Inhibition of Tyrosine Kinase Activity of HER-2/neu Receptor.**

 In an initial round of *in vivo* trials, inventors will use a mouse model of human cancer with the histologic features and metastatic potential resembling tumors seen in
10 humans (Katsumata *et al.*, 1995) and treat these animals with emodin and or emodin-like compound to examine the suppression of tumor development.

 These studies are based on the discovery that emodin is an inhibitor for the *neu* tyrosine kinase receptor and functions as a tumor suppressor for *neu*-overexpressing
15 cancer cells (Examples 2-6). The Examples above further show that emodin inhibits the growth of *neu*-mediated cancer cells and furthermore sensitizes *neu*-mediated cancer cells to chemotherapeutic drugs. The current example uses of both *neu* tyrosine kinase inhibitors, such as emodin alone or in combination with chemotherapeutic drugs, to provide a useful preventive and therapeutic regimen for
20 patients with *neu*-overexpressing cancers.

 Two groups of mice of a suitable cancer model will be treated with doses of emodin or emodin-like compounds either alone or in combination with anti cancer drugs starting at 6 weeks of age. Several combinations and concentrations of emodin,
25 emodin-like compounds and anti-cancer drugs will be tested. Control mice will be treated with buffer only.

 The effect of emodin or emodin-like compounds on the development of breast tumors will be compared with the control group by examination of tumor size. p185^{neu}

tyrosine kinase activity (using IP-western blot analysis) and histopathologic examination (breast tissue will be cut and stained with hematoxylin and eosin) of breast tissue. With the chemopreventive potential of emodin or emodin-like compounds, it is predicted that, unlike the control group of mice that develop tumors, the testing group of mice will be resistant to tumor development.

Breast Cancer Model

In an exemplary study, emodin was tested in mice, at 40 mg/kg, 3 days a week for 2 weeks, the mice did not show any weight loss. Although human breast cancer cells differ from murine cancer cells, the inventors will use a similar approach to treat animal carrying *HER-2/neu*-overexpressing human breast cancers. Emodin dosage will escalate from 40 mg/kg to 60 mg/kg, 80 mg/kg, and 100 mg/kg. To accomplish this, mice will be injected in m.f.p. with 0.1 ml ($1-10 \times 10^6$) of tumorigenic breast cancer cell suspension (*HER-2/neu*-overexpressing MDA-MB 361 and BT 474 cells; MDA-MB 435 and MDA-MB 231 cells which express normal levels of *HER-2/neu* expression). The mice will be randomly assigned to untreated control (1 group) and drug-treated groups (4 groups) of 6 mice/group (total, 30 mice for each cell line). The animals will be weighed weekly and tumor volumes will be determined with calipers using the formulation: $\text{volume (mm)}^3 = \text{width (mm)}^2 \times \text{length (mm)} / 2$ (Giovannella *et al.*, 1982). When palpable tumor nodules (larger than 2 mm^3) can be detected, the tumor-bearing mice will be treated i.p. with 0.2 ml of placebo [chromophore/DMSO/saline (1:2:7)] or emodin: at the dosage of (a) 40 mg/kg, (b) 60 mg/kg, (c) 80 mg/kg, (d) 100 mg/kg, 3 days a week. The pharmacokinetics of emodin in rabbits were recently reported: the elimination half-life of emodin was 227 min. and oral administration of emodin resulted in very low serum concentration. Although mice may be different from rabbits in emodin metabolism, i.p. injection is a preferred method of administration because it was effective in the preliminary studies. The animals' tumor volumes will be monitored once a week, and survival curves will be

drawn. Responses to emodin will be quantified as changes in tumor volume. Using median values obtained in treated and control animals, statistical significance will be determined by the Wilcoxon rank sum test (Dawson-Saunders and Trapp, 1990). Overall toxicity will be measured as percentage of body weight loss and death. The tumor inhibitory effects of emodin in the two breast tumor systems (i.e. *HER-2/neu* overexpressing or non-overexpressing) will be compared. These studies will thus allow the determination of an optimal dose of emodin with maximum antitumor activity and lowest toxicity. This schedule and dose will be used for any subsequent administration.

10 In the inventors previous studies shown above, both MDA-MB-361 (*HER-2/neu* overexpression) and MDA-MB-435 (normal levels of *HER-2/neu*) cell lines produced spontaneous metastases after inoculation into m.f.p. These two breast cancer cell lines will be inoculated into the m.f.p. of mice (15 mice for each line, total of 30 mice). Three months later, 3 mice inoculated with each cell line will be sacrificed to examine the formation of metastatic tumors. The remaining mice inoculated with each cell line will be divided into 2 groups (6 mice/group). One group will be treated with emodin at optimal conditions obtained as above; the control group will be given placebo under the same conditions. Treatment will continue for 3 more months, then the mice will be sacrificed and distant metastasis sites will be examined.

Ovarian Cancer Model

25 In order to obtain mice having human ovarian cancer, *nu/nu* mice may be given intraperitoneal injections of, for example, 2×10^6 viable p185-overexpressing SKOV-3 human ovarian cancer cells. Mice sacrificed 5 days post treatment exhibit tumors resulting from such treatment.

Five days after treatment with the p185-overexpressing cells, mice may be separated into control and experimental groups. One group of mice will be left

untreated. Other groups will be treated. Active compounds may be supplied to a treated group in phosphate buffer saline. One treated group will be treated with the buffered saline only. Another treated group may receive treatment with an appropriate dosage of emodin or an emodin-like compound alone. A third treated group may be treated with an appropriate dosage of an anti-cancer drug alone. A final group may be treated with an appropriate dosage of emodin or an emodin-like compound in combination with an anti-cancer drug. Treatments may be given intraperitoneally.

10 Mice may be examined for tumor signs and symptoms, and killed when they appear moribund. Mice treated with the emodin or emodin-like compound plus the anti-cancer drug will be expected to have a longer survival time.

Small Cell Lung Cancer Model

15 In order to obtain mice with the human lung cell cancer, *nu/nu* mice may be given as intratracheal injections of, for example, 2×10^6 viable *neu* overexpressing cancer cells from cell line H82. Five days after inoculation, following tumor formation, mice may be separated into groups to begin treatment. One group may be treated with an appropriate dosage of emodin or an emodin-like compound alone.
20 another with an appropriate dosage of an anti-cancer drug alone. A third group may be treated with an appropriate dosage of emodin or an emodin-like compound in combination with an emodin for 3 consecutive days, then once a week for two months.

EXAMPLE 14**Model to test the Effect of Emodin and chemotherapeutic drugs on
HER2/neu overexpressing breast cancer in vitro and in vivo.**

5 Drugs such as paclitaxel, cyclophosphamide, and doxorubicin are currently
used for treatment of breast cancer, but these drugs are less effective for patients with
HER-2/neu overexpressing breast cancers. The inventors will test paclitaxel for
chemosensitivity of breast cancer cell lines by combined treatment with emodin or
emodin-like compound, because paclitaxel has been used for patients who have
10 advanced metastatic breast cancer and have failed prior chemotherapy (Chevalier *et al.*, 1995; Klaassen *et al.*; Gianna *et al.*, 1994; Chang *et al.*, 1995), and *HER-2/neu*
overexpressing is implicated in paclitaxel resistance in vitro (Yu *et al.*, 1996).

Effect of emodin and paclitaxel in combination on growth of breast cancer
15 cells MDA-MB 361, BT 474, MDA-MB 435, and MDA-MB 231 *in vitro*. Using a
similar protocol used on lung cancer cells (Zhang and Hung, 1996) for this study.
Breast cancer cells will be first treated with various concentrations of emodin (1, 10,
20, 40, 60, 80, and 100 μ M) alone or paclitaxel (1, 10, and 100 nM, 1, 10, 50, and 100
 μ M) alone for 12, 24, 48, and 72 hour. The cell growth rate will be determined by the
20 MTT assay (Zhang *et al.*, 1995), and the number of viable cells will be determined by
trypan-blue staining and visual counting of samples in a hemocytometer (Zhang *et al.*,
1991). For each time point and drug treatment, a minimum of three plates will be
counted. In addition, the rate of DNA synthesis of these cells will be determined in
parallel plates by the [3 H]thymidine incorporation assay (Zhang *et al.*, 1994). The
25 effect of emodin and paclitaxel on the ability of forming colonies on soft agar of
breast cancer cells will be determined by soft-agar assay (Zhang *et al.*, 1995). The
results will be used to establish the dose-response curves and at various exposure
times to critically determine the minimum toxic dose range of the drug and the most
efficacious duration of treatment for subsequent studies. Thus, various inhibitory

concentrations (ICs) for each drug can be determined. To determine whether the combinations exert a synergistic effect, different ratios of emodin and paclitaxel will be added simultaneously at specific inhibitory levels, e.g., IC₂₅ + IC₂₅, IC₅₀ + IC₅₀, and IC_{12.5} + IC₇₅ (in both combinations). To evaluate the combined effect of the two drugs, the observed values will be compared to the predicted values [c] calculated from the equation $[c] = [a] \times [b]/100$, where [a] and [b] indicate survival values with single agents; observed values of less than 70% of predicted ones will be considered synergistic (Zhang and Hung, 1996; Hata *et al.*, 1994).

Then, preclinical animal experiments will be used to measure the therapeutic efficacy of emodin and emodin-like compounds. The orthotopic breast cancer models (m.f.p. tumor model, MDA-MB 361, BT 474, MDA-MB 435, and MDA-MB 231 will be used to induce tumor at m.f.p. of BALB/c nude mice) described above will be used to test the ability of emodin to sensitize to paclitaxel. When palpable tumor nodules (larger than 2 mm³) can be detected, the tumor-bearing mice will be given emodin i.p. 3 days a week, the minimum dose of emodin that maximizes efficacy and minimizes toxicity, will be based on results from Example 13. Paclitaxel has been shown to inhibit tumor growth in nude mice bearing subcutaneous human mesotheliomas (Lee *et al.*, 1995) and human lung carcinoma MV 522 xenografts (Kelner *et al.*, 1995) under conditions, respectively, of i.p. injection of 30 mg/kg, 3 times a week (Lee *et al.*, 1995), and 10 mg/kg, of i.p. injection 5 days a week for 3 cycles (Kelner *et al.*, 1995). Based on this, the inventors treat mice bearing breast tumor (6 mice/group) with paclitaxel alone (5, 10, 20, and 30 mg/kg body weight; for control, the same volume of placebo) in i.p. injection 3 times a week. Then 20%, 50%, and 75% tumor inhibitory doses of paclitaxel will be combined with emodin (optimal dose obtained from work for Example 13) and administered i.p. 3 times a week to breast tumor-bearing mice. Tumor size and mouse weight will be measured once a week, calculation of tumor volume and synergistic effect, and overall evaluation of efficacy

and toxicity will be done with the methods described for Example 13. From these evaluations the inventors expect the paclitaxel dose required to reduce tumor volume in the *HER-2/neu*-overexpressing animals to be significantly lower when combined with emodin than the dose of paclitaxel alone, and the *HER-2/neu* low-expressing
5 animals not to be sensitized to paclitaxel by emodin.

In yet a further the study the inventors propose to determine whether emodin and emodin like compounds prevent breast tumor development by using *neu*-transgenic mice. The transgenic mice [transgenic FVB/N-TgN (MMTV*neu*) 202]
10 that express the *HER-2/neu* oncogene (*neuT*) in mammary epithelial cells and develop breast tumors at the mean age of 44 weeks old (comparable to human middle age) are purchased from Jackson Laboratory (Bar Harbor, Marion), and used to examine whether emodin suppresses mammary tumor development. Beginning at 6 weeks of age, these mice (15 mice/group) will be treated with emodin by i.p. injection
15 of 5 different doses (starting at 20 mg/kg of body weight, then rise to 40 mg/kg, 60 mg/kg, 80 mg/kg, and 100 mg/kg, respectively) 3 times a week. Control transgenic mice will be treated with placebo only. The effect of emodin on the development of breast tumors will be compared in the treated and control groups by examining tumor size. p185^{*neu*} tyrosine kinase activity (using immunocomplex assay), tyrosine
20 phosphorylation level of p185^{*neu*} (using immunoprecipitation-western blot analysis) (Zhang *et al.*, 1995), and histopathological examination (breast tissue will be cut and stained with hematoxylin and eosin). According to the present invention, the chemopreventive potential of emodin becomes evident when the tested group will resist breast tumor development.

25

EXAMPLE 14**Human Treatment with Emodin-Like Tyrosine Kinase Inhibitors in
Combination with Anti-cancer Drugs or Alone**

5 This example describes a protocol to facilitate the treatment of *neu*-mediated cancer using emodin or an emodin-like tyrosine kinase inhibitor alone or in combination with anti-cancer drugs.

10 A patient presenting a *neu*-mediated cancer may be treated using the following protocol. *Neu*-overexpression may be detected using the immunohistochemistry methods described below. Patients may but need not have received previous chemo-radio- or gene therapeutic treatments. Optimally the patient will exhibit adequate bone marrow function (defined as peripheral absolute granulocyte count of > 2,000/mm³ and platelet count of 100, 000/mm³, adequate liver function (bilirubin
15 1.5mg/dl) and adequate renal function (creatinine 1.5mg/dl).

Monitoring *neu* overexpression in tumors

 The over-expression of *neu* is typically monitored before, during, and after the therapy. The following assay may be used to monitor *neu*-overexpression. Sections of
20 3- to 4 mm thickness of the primary tumors and of the cell block preparations are cut, deparaffinized in xylene, and rehydrated in descending grades (100-70%) of ethanol. Endogenous peroxidase activity is blocked with 3% hydrogen peroxide in methanol. After several washes in distilled water and phosphate-buffered saline, the sections are incubated with a 1:10 dilution of normal horse serum to minimize background staining.
25 This is followed by incubation for 1 hr at room temperature with the primary antibody (Ab-3 monoclonal antibody, Oncogene Sciences, Uniondale, NY; 1:100). The peroxidase staining procedure utilizes ABC Elite Kits (Vector Laboratories, Burlingame, CA). The immunostaining reactions are visualized using 3-amino-9-ethylcarbazole as the chromogen. The sections and/or cytospin preparations are stained with toluidine blue

and mounted in permount. Positive and negative control immunostains are also prepared.

5 The sections are reviewed by the pathologist. Two features of the immunoreaction will be recorded using a semi quantitative scale: the relative number of positive cells (0%, <10%, 10-50%, and > 50%) and the intensity of the reaction (0-3). The pattern of immunostaining (membranous, cytoplasmic) is recorded separately. A tumor is considered *neu* positive if any neoplastic cells show cell membrane reactivity. Cytoplasmic staining is considered non-specific. A breast carcinoma known for its
10 strong positive membrane staining will be used as a positive control.

The quantitative measurement of *neu* immunostaining will be performed using computerized image analysis with the SAMBA 4000 Cell Image Analysis System (Image Products International, Inc., Chantilly, VA) integrated with a Windows based
15 software. A strong staining tumor tissue section will be used as positive control. The primary antibody will be replaced by an isotype-matched irrelevant antibody to set the negative control threshold, averaging the results from ten fields.

Protocol for the Treatment of *neu*-Mediated Cancer

20 A composition of the present invention is typically administered orally or parenterally in dosage unit formulations containing standard, well known non-toxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intra-arterial injection, or infusion techniques. The emodin or
25 emodin-like compound may be delivered to the patient before, after or concurrently with the other anti-cancer agents.

A typical treatment course may comprise about six doses delivered over a 7 to 21 day period. Upon election by the clinician the regimen may be continued six doses

every three weeks or on a less frequent (monthly, bimonthly, quarterly etc.) basis. Of course, these are only exemplary times for treatment, and the skilled practitioner will readily recognize that many other time-courses are possible.

5 A major challenge in clinical oncology of *neu*-mediated cancers is that tumor cells over-expressing the *neu*-protooncogene are resistant to chemotherapeutic treatment. One goal of the inventors' efforts has been to find ways to improve the efficacy of chemotherapy. In the context of the present invention, emodin or an emodin-like compound can be combined with any of a number of conventional
10 chemotherapeutic regimens.

 To kill *neu*-overexpressing cancer cells using the methods and compositions described in the present invention one will generally contact a target cell with emodin or an emodin like tyrosine kinase inhibitor and at least one chemotherapeutic agent
15 (second agent), examples of which are described above. These compositions will be provided in a combined amount effective to kill or inhibit the proliferation of the cell. This process may involve contacting the cell with emodin or emodin-like compounds and the second agent at the same time. Alternatively, this process may involve contacting the cell with a single composition or pharmacological formulation that
20 includes both agents or by contacting the cell with two distinct compositions or formulations at the same time, wherein one composition includes the emodin or emodin-like tyrosine kinase inhibitor and the other includes the second agent.

 Alternatively the emodin or emodin-like compound administration may
25 precede or follow the delivery of the second agent by intervals ranging from minutes to weeks. In embodiments where the emodin or emodin-like tyrosine kinase inhibitor and the second compound are applied separately, one would ensure that a significant period of time did not expire between the time of each delivery, such that the second agent and the emodin or emodin-like compound would still be able to exert an

advantageously combined effect on the cancer. In such instances, it is contemplated that one would contact the cell with both agents within about 6 hours to one week of each other and more preferably, within 24-72 hours of each other. In some situations however, it may be desirable to extend the time period for treatment significantly where several days (2, 3, 4, 5, 6, 7 or more) to several weeks (1, 2, 3, 4, 5, 6, 7 or more) lapse between respective administrations.

Regional delivery of emodin or emodin-like tyrosine kinase inhibitors will be an efficient method for delivering a therapeutically effective dose to counteract the clinical disease. Likewise, the chemotherapy may be directed to a particular effected region. Alternatively systemic delivery of either, or both, agent may be appropriate.

The therapeutic composition of the present invention is administered to the patient directly at the site of the tumor. This is in essence a topical treatment of the surface of the cancer. The volume of the composition should usually be sufficient to ensure that the entire surface of the tumor is contacted by the emodin or emodin like compound and second agent.

In one embodiment, administration simply entails injection of the therapeutic composition into the tumor. In another embodiment, a catheter is inserted into the site of the tumor and the cavity may be continuously perfused for a desired period of time.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by the disappearance of all measurable disease for at least a month. Whereas a partial response may be defined by a 50% or greater reduction of the sum of the products of perpendicular diameters of all evaluable tumor nodules or at least 1 month with no tumor sites showing enlargement. Similarly, a mixed response may be defined by a reduction of the product of perpendicular

diameters of all measurable lesions by 50% or greater with progression in one or more sites.

Of course, the above-described treatment regimes may be altered in accordance with the knowledge gained from clinical trials such as those described in Example 14. Those of skill in the art will be able to take the information disclosed in this specification and optimize treatment regimes based on the clinical trials described in the specification.

10

EXAMPLE 16

Clinical Trials of the Use of Tyrosine Kinase Inhibitors in Combination with Anti-cancer Drugs or Alone in Treating *Neu*-Mediated Cancer

This example is concerned with the development of human treatment protocols using the emodin and emodin-like compound alone or in combination with anti-cancer drugs. Emodin or emodin-like compounds and anti-cancer drug treatment will be of use in the clinical treatment of various *neu*-overexpressing cancers in which transformed or cancerous cells play a role. Such treatment will be particularly useful tools in anti-tumor therapy, for example, in treating patients with ovarian, breast and lung cancers that are mediated by *neu* over-expression and resistant to conventional chemotherapeutic regimens.

The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for use in establishing emodin or emodin-like compounds alone or in combinations with anti-cancer drugs in clinical trials.

Patients with advanced, metastatic breast and/or epithelial ovarian carcinoma chosen for clinical study will typically have failed to respond to at least one course of conventional therapy. Measurable disease is not required, however the patient must have easily accessible pleural effusion and/or ascites. Further the patients must carry tumors that overexpress *neu* oncoprotein. Overexpression may be defined as grade 2 or 3 staining by immunohistochemistry as described above. In an exemplary clinical protocol, patients may undergo placement of a Tenckhoff catheter, or other suitable device, in the pleural or peritoneal cavity and undergo serial sampling of pleural/peritoneal effusion. Typically, one will wish to determine the absence of known loculation of the pleural or peritoneal cavity, creatinine levels that are below 2 mg/dl, and bilirubin levels that are below 2 mg/dl. The patient should exhibit a normal coagulation profile.

In regard to the emodin or emodin-like compounds and other anti-cancer drug administration, a Tenckhoff catheter, or alternative device may be placed in the pleural cavity or in the peritoneal cavity, unless such a device is already in place from prior surgery. A sample of pleural or peritoneal fluid can be obtained, so that baseline cellularity, cytology, LDH, and appropriate markers in the fluid (CEA, CA15-3, CA 125, p185) and in the cells (E1A, p185) may be assessed and recorded..

20

In the same procedure, emodin or emodin-like compound may be administered alone or in combination with the anti-cancer drug. The administration may be in the pleural/peritoneal cavity, directly into the tumor, or in a systemic manner. The starting dose may be 0.5mg/kg body weight. Three patients may be treated at each dose level in the absence of grade ≥ 3 toxicity. Dose escalation may be done by 100% increments (0.5mg, 1mg, 2mg, 4mg) until drug related grade 2 toxicity is detected. Thereafter dose escalation may proceed by 25% increments. The administered dose may be fractionated equally into two infusions, separated by six hours if the combined endotoxin levels

25

determined for the lot of emodin or emodin-like compound and the lot of anti-cancer drug exceed 5EU/kg for any given patient.

5 The emodin or emodin-like compound and anti-cancer drug combination may be administered over a short infusion time or at a steady rate of infusion over a 7 to 21 day period. The emodin and/or emodin-like compound infusion may be administered alone or in combination with the anti-cancer drug. The infusion given at any dose level will be dependent upon the toxicity achieved after each. Hence, if Grade II
10 toxicity was reached after any single infusion, or at a particular period of time for a steady rate infusion, further doses should be withheld or the steady rate infusion stopped unless toxicity improved. Increasing doses of emodin and/or emodin-like compound alone or in combination with an anti-cancer drug will be administered to groups of patients until approximately 60% of patients showed unacceptable Grade III or IV toxicity in any category. Doses that are 2/3 of this value could be defined as the
15 safe dose.

Physical examination, tumor measurements, and laboratory tests should, of course, be performed before treatment and at intervals of about 3-4 weeks later. Laboratory studies should include CBC, differential and platelet count, urinalysis,
20 SMA-12-100 (liver and renal function tests), coagulation profile, and any other appropriate chemistry studies to determine the extent of disease, or determine the cause of existing symptoms. Also appropriate biological markers in serum should be monitored e.g. CEA, CA 15-3, p185 and p185^{neu} tyrosine phosphorylation for breast cancer, and CA 125, p185 tyrosine phosphorylation for ovarian cancer

25

To monitor disease course and evaluate the anti-tumor responses, it is contemplated that the patients should be examined for appropriate tumor markers every 4 weeks, if initially abnormal, with twice weekly CBC, differential and platelet count for the 4 weeks; then, if no myelosuppression has been observed, weekly. If any

patient has prolonged myelosuppression. a bone marrow examination is advised to rule out the possibility of tumor invasion of the marrow as the cause of pancytopenia. Coagulation profile shall be obtained every 4 weeks. An SMA-12-100 shall be performed weekly. Pleural/peritoneal effusion may be sampled 72 hours after the first
5 dose, weekly thereafter for the first two courses, then every 4 weeks until progression or off. study. Cellularity, cytology, LDH, and appropriate markers in the fluid (CEA, CA15-3, CA 125, p185 tyrosine phosphorylation) and in the cells (p185 tyrosine phosphorylation) may be assessed. For an example of an evaluation profile, see Table 3. When measurable disease is present, tumor measurements are to be recorded every 4
10 weeks. Appropriate radiological studies should be repeated every 8 weeks to evaluate tumor response. Spirometry and DLCO may be repeated 4 and 8 weeks after initiation of therapy and at the time study participation ends. An urinalysis may be performed every 4 weeks.

15 Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by the disappearance of all measurable disease for at least a month. Whereas a partial response may be defined by a 50% or greater reduction of the sum of the products of perpendicular diameters of all evaluable tumor nodules or at least 1 month with no tumor sites showing enlargement. Similarly, a
20 mixed response may be defined by a reduction of the product of perpendicular diameters of all measurable lesions by 50% or greater with progression in one or more sites.

Table 3
EVALUATIONS BEFORE AND DURING THERAPY

| EVALUATIONS | PRE-STUD Y | TWICE WEEKLY | WEEKLY | EVERY 4 WEEKS | EVERY 8 WEEKS |
|---|---------------|-----------------|----------------|------------------|------------------|
| History | X | | | X | |
| Physical | X | | | X | |
| Tumor Measurements | X | | | X | |
| CBC | X | X ¹ | X | | |
| Differential | X | X ¹ | X | | |
| Platelet Count | X | X ¹ | X | | |
| SMA12-100 (SGPT, Alkaline Phosphatase, Bilirubin, Alb/Total Protein) | X | | X | | |
| Coagulation Profile | X | | | X | |
| Serum Tumor markers (CEA, CA15-3, CA-125, Her-2/neu tyrosine phosphorylation) | X | | | X ³ | |
| Urinalysis | X | | | X | |
| X-rays: | | | | | |
| chest | X | | X ⁴ | | |
| others | X | | | | X |
| Pleural/Peritoneal Fluids: (cellularity, cytology, LDH, tumor markers, level of HER-2/neu tyrosine phosphorylation) | X | | X ⁵ | X | |
| Spirometry and DLCO | X | | | X ⁶ | X ⁶ |

¹ For the first 4 weeks, then weekly, if no myelosuppression is observed.

5 ² As indicated by the patient's condition.

³ Repeated every 4 weeks if initially abnormal.

⁴ For patients with pleural effusion, chest X-rays may be performed at 72 hours after first dose, then prior to each treatment administration.

10 ⁵ Fluids may be assessed 72 hours after the first dose, weekly for the first two courses and then every 4 weeks thereafter.

⁶ Four and eight weeks after initiation of therapy.

* * *

5 All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

1. A method of inhibiting transformation of a cell, comprising contacting the cell with an emodin-like tyrosine kinase inhibitor and a chemotherapeutic drug in amounts
5 effective to inhibit the transformed phenotype.
2. The method of claim 1, wherein the transformation is *neu* oncogene-mediated transformation.
10
3. The method of claim 1, wherein the cell comprises a tyrosine specific protein kinase encoded by *neu*.
15
4. The method of claim 1, wherein the emodin-like tyrosine kinase inhibitor has a chemical structure of DK-III-8; DK-III-19; DK-III-47; DK-III-48; DK-III-13; DK-III-11; DK-II-1; DK-II-2; DK-IV-1; DK-V-47; DK-V-48; DK-III-52 as set forth in Table 1.
20
5. The method of claim 1, wherein the emodin-like tyrosine kinase inhibitor is an anthraquinone tyrosine kinase inhibitor.
- 25 6. The method of claim 1, wherein the emodin-like tyrosine kinase inhibitor is emodin.

7. The method of claim 1, wherein the cell is contacted with between about
0.5mg/kg total weight and 500mg/kg total weight of the emodin-like tyrosine kinase
5 inhibitor.
8. The method of claim 1, wherein the cell is contacted with between about
0.5mg/kg total weight and 500mg/kg total weight emodin.
10
9. The method of claim 1, wherein the chemotherapeutic is an alkylating agent.
- 15 10. The method of claim 9, wherein the alkylating agent is mechlorethamine,
cyclophosphamide, ifosfamide chlorambucil, melphalan, busulfan, thiotepa,
carmustine, lomustine, or shreptozoin.
- 20 11. The method of claim 1, wherein the chemotherapeutic agent comprises a plant
alkaloid.
- 25 12. The method of claim 11, wherein the plant alkaloid is vincristine, vinblastine
or taxol.

13. The method of claim 12, wherein the plant alkaloid is taxol.

14. The method of claim 1, wherein the chemotherapeutic agent is an antibiotic.

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15. The method of claim 14, wherein the antibiotic is dactinomycin, daunorubicin, idarubicin, bleomycin, mitomycin, or doxorubicin.

10

16. The method of claim 15, wherein the antibiotic is doxorubicin.

17. The method of claim 1, wherein the chemotherapeutic drug is an antineoplastic.

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18. The method of claim 17, wherein the antineoplastic agent is selected from the group consisting of cisplatin, VP16, and TNF.

20

19. The method of claim 1, wherein the emodin-like tyrosine kinase inhibitor and the chemotherapeutic drug are administered substantially simultaneously.

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20. The method of claim 1, wherein the cell is located within an animal and effective amounts of the emodin-like tyrosine kinase inhibitor and the chemotherapeutic drug are administered to the animal.
- 5
21. The method of claim 1, wherein the emodin-like tyrosine kinase inhibitor is suitably dispersed in a pharmacologically acceptable formulation.
- 10
22. The method of claim 1, wherein the cell is contacted with a single composition comprising an emodin-like tyrosine kinase inhibitor in combination with a chemotherapeutic agent.
- 15
23. The method of claim 22, wherein the composition is suitable the composition is suitably dispersed in a pharmacologically acceptable formulation.
24. The method of claim 1, wherein the cell is a human cell.
- 20
25. The method of claim 24, wherein the cell is a lung cancer cell.
- 25
26. The method of claim 24, wherein the cell is a breast cancer cell.

27. A method of sensitizing a cancer cell to a chemotherapeutic drug comprising exposing the cell with an amount of emodin-like inhibitor effective to inhibit *neu*-mediated tyrosine kinase activity in the cell.

5

28. A method of inhibiting cancer comprising administering to an animal having or suspected of having cancer an effective combination of emodin-like tyrosine kinase inhibitor and chemotherapeutic drug in an effective amount to inhibit the cancer.

10

29. The method of claim 28, wherein the animal is a human.

30. The method of claim 28 comprising injecting into the animal therapeutically effective amounts of an emodin-like tyrosine kinase inhibitor and contacting the animal with a chemotherapeutic drug.

15

31. The method of claim 28, wherein the emodin-like tyrosine kinase inhibitor is emodin.

20

32. The method of claim 28, wherein a cancer site is contacted with a chemotherapeutic drug by administering to the animal a therapeutically effective amount of a pharmaceutical composition comprising a chemotherapeutic drug.

25

33. The method of claim 28, wherein the chemotherapeutic drug is cisplatin, doxorubicin, or VP16.
- 5 34. A pharmaceutical composition comprising an emodin-like tyrosine kinase inhibitor and a chemotherapeutic drug.
- 10 35. A composition of claim 34, wherein the chemotherapeutic drug is cisplatin, doxorubicin, VP16, taxol, or TNF.
- 15 36. The composition of claim 34, wherein the emodin-like tyrosine kinase inhibitor is emodin.
- 20 37. A pharmaceutical combination comprising an emodin-like tyrosine kinase inhibitor and a chemotherapeutic drug.
- 25 38. The pharmaceutical combination of claim 37, wherein the emodin-like tyrosine kinase inhibitor is an anthraquinone-like tyrosine kinase inhibitor.
39. The pharmaceutical combination of claim 37, wherein the emodin-like tyrosine kinase inhibitor is emodin.

40. The pharmaceutical combination of claim 37, wherein the chemotherapeutic drug is cisplatin, doxorubicin, VP16, taxol, or TNF.

5

41. The pharmaceutical combination of claim 37, wherein the emodin-like tyrosine kinase inhibitor and the chemotherapeutic drug are comprised in the same pharmaceutical composition.

10

42. A therapeutic kit comprising in suitable container, a pharmaceutical formulation of an emodin-like tyrosine kinase inhibitor, and a pharmaceutical formulation of a chemotherapeutic drug.

15

43. A kit of claim 42, wherein the pharmaceutical formulation of an emodin-like tyrosine kinase inhibitor and the pharmaceutical formulation of a chemotherapeutic drug are present in the same container.

20

44. A kit of claim 42, wherein the pharmaceutical formulation of an emodin-like tyrosine kinase inhibitor and the pharmaceutical formulation of a chemotherapeutic drug are present within distinct containers.

25

45. A method of inhibiting transformation of a cell, comprising contacting the cell with an emodin-like tyrosine kinase inhibitor in an amount effective to inhibit the transformed phenotype.

5

46. The method of claim 45, wherein the transformation is *neu* oncogene-mediated transformation.

10

47. The method of claim 45, wherein the emodin-like tyrosine kinase inhibitor has a chemical structure of DK-III-8; DK-III-19; DK-III-47; DK-III-48; DK-III-13; DK-III-11; DK-II-1; DK-II-2; DK-IV-1; DK-V-47; DK-V-48; DK-III-52 as set forth in Table 1.

15

48. The method of claim 45, wherein the emodin-like tyrosine kinase inhibitor is, emodin-8-O-D-glucoside, chrysophanic acid, gluco-chrysophanic acid, physcion, physcion-8-O-D-glucoside, or an emodin-like tyrosine kinase inhibitor.

20

49. The method of claim 45, wherein the emodin-like tyrosine kinase inhibitor is an anthraquinone tyrosine kinase inhibitor.

25

50. The method of claim 45, where in the emodin-like tyrosine kinase inhibitor is emodin.

51. The method of claim 45, wherein the cell is contacted with between about 0.5mg/kg total weight and about 500mg/kg total weight of the emodin-like tyrosine kinase inhibitor.

5

52. The method of claim 45, wherein the cell is contacted with between about 0.5mg/kg total weight and about 500mg/kg total weight emodin.

FIG. 1B

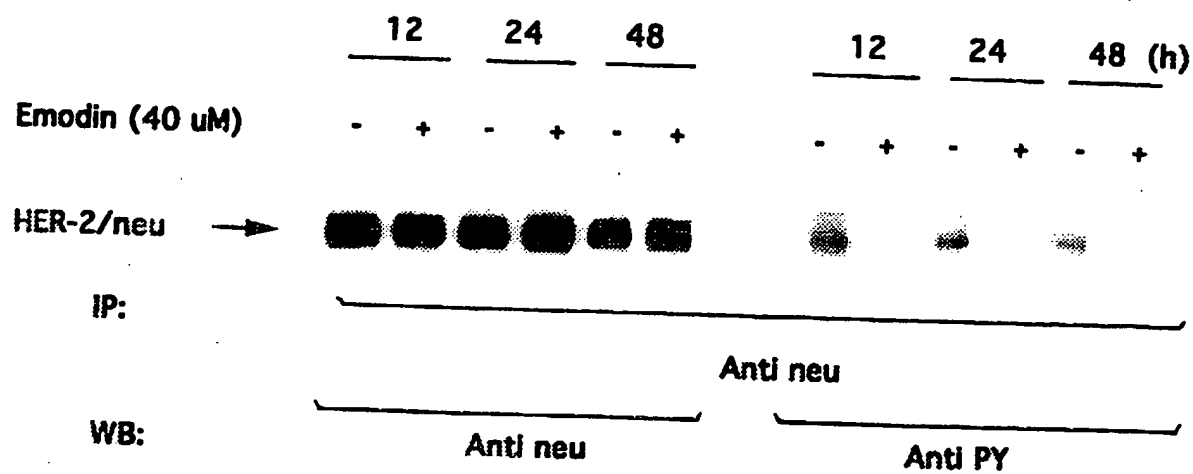


FIG. 2A

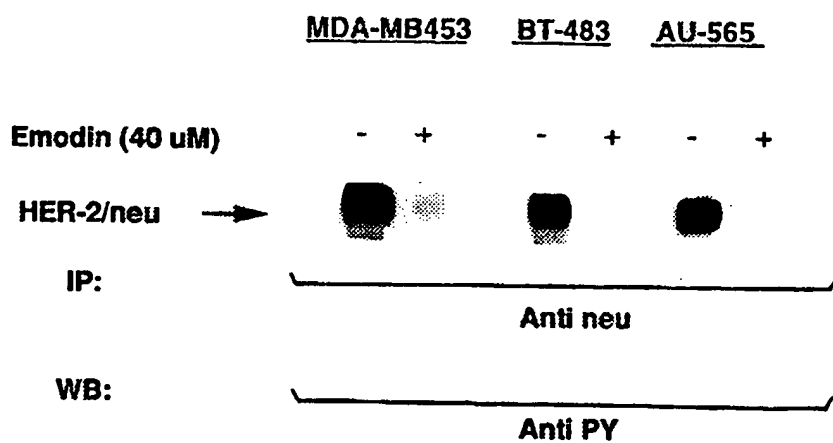


FIG. 2B

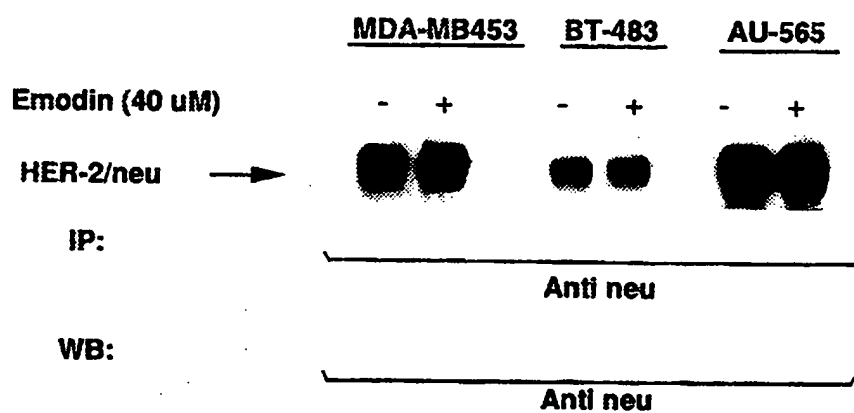


FIG. 3A

A.

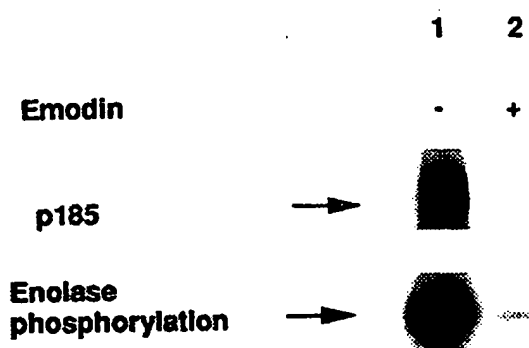
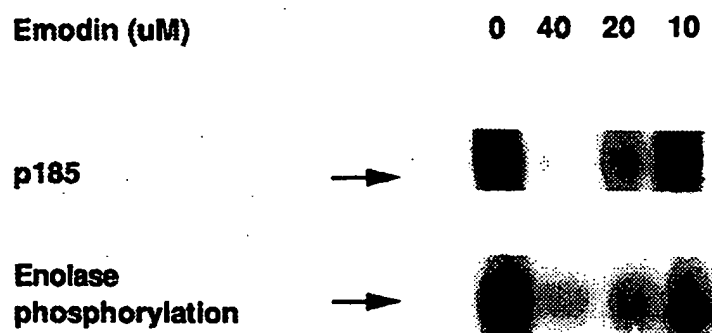


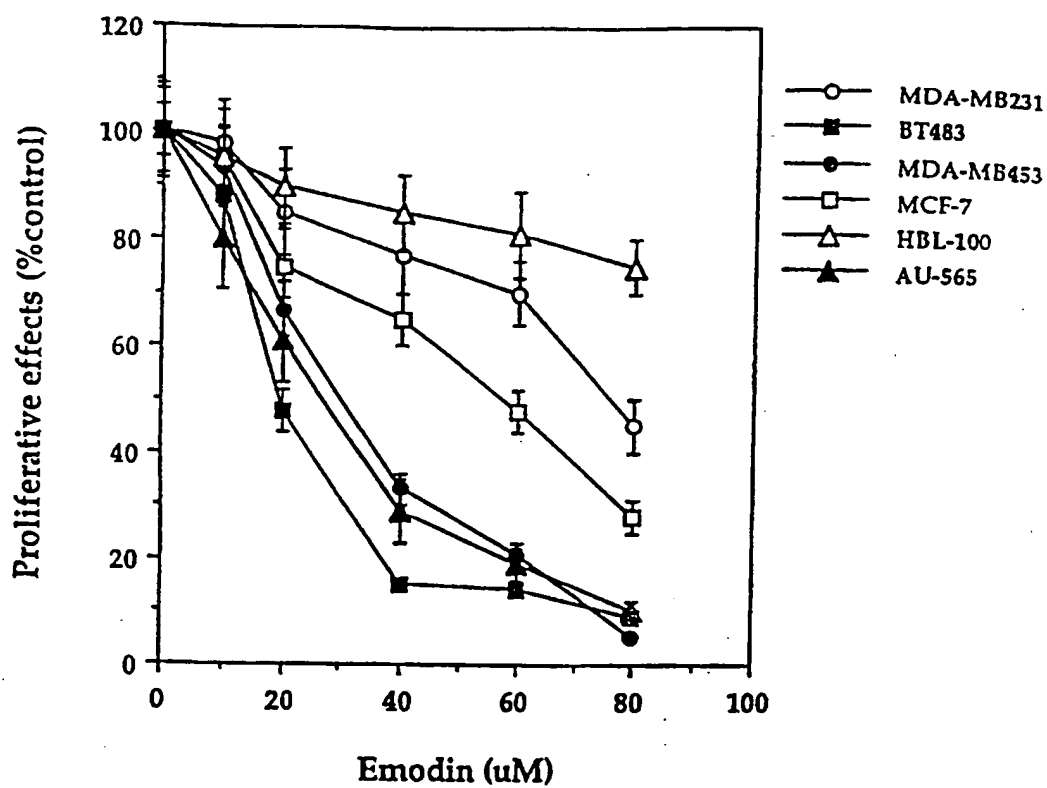
FIG. 3B

B.



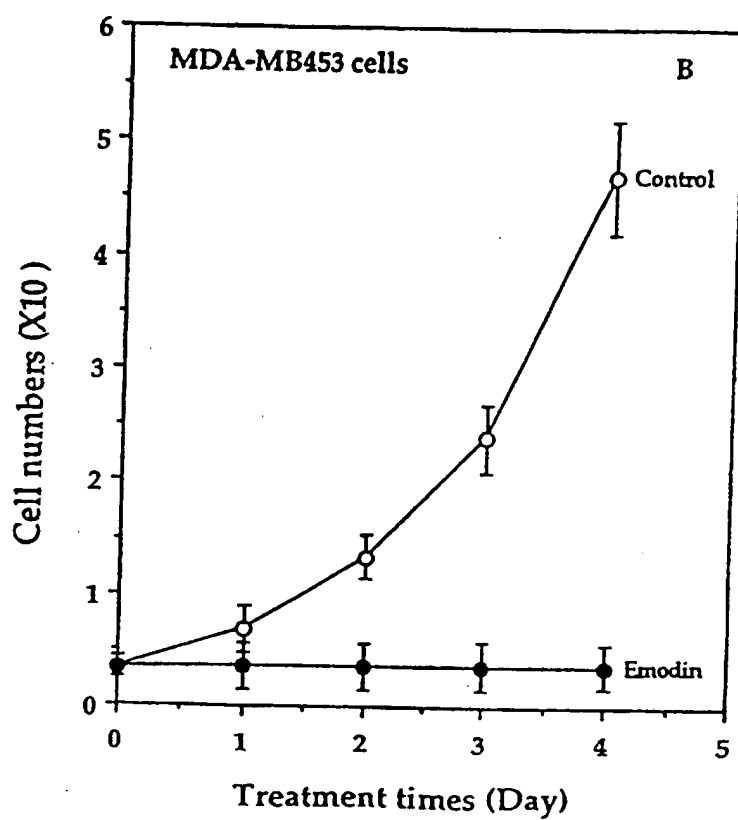
7/35

FIG. 4A



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FIG. 4B



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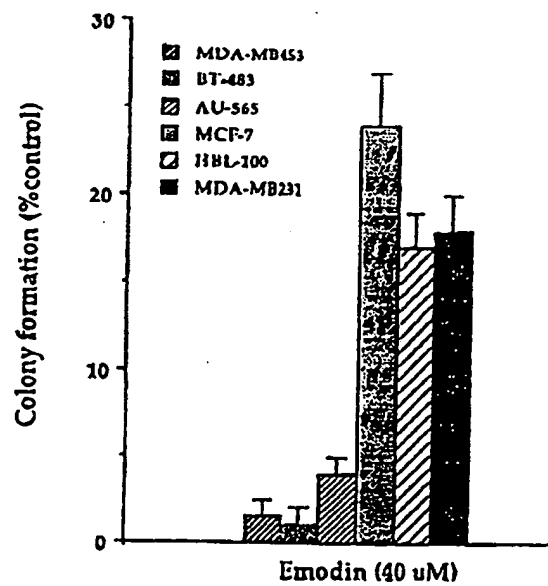


FIG. 5

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FIG. 6A

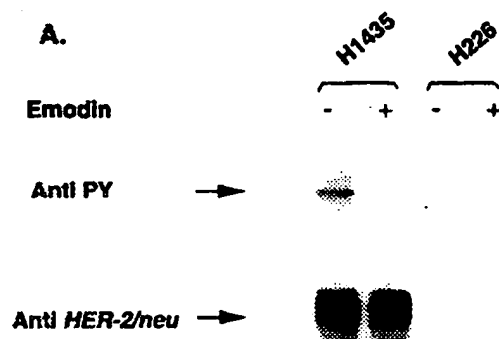
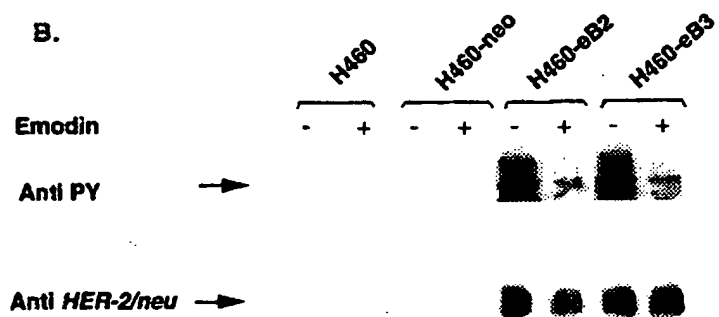


FIG. 6B



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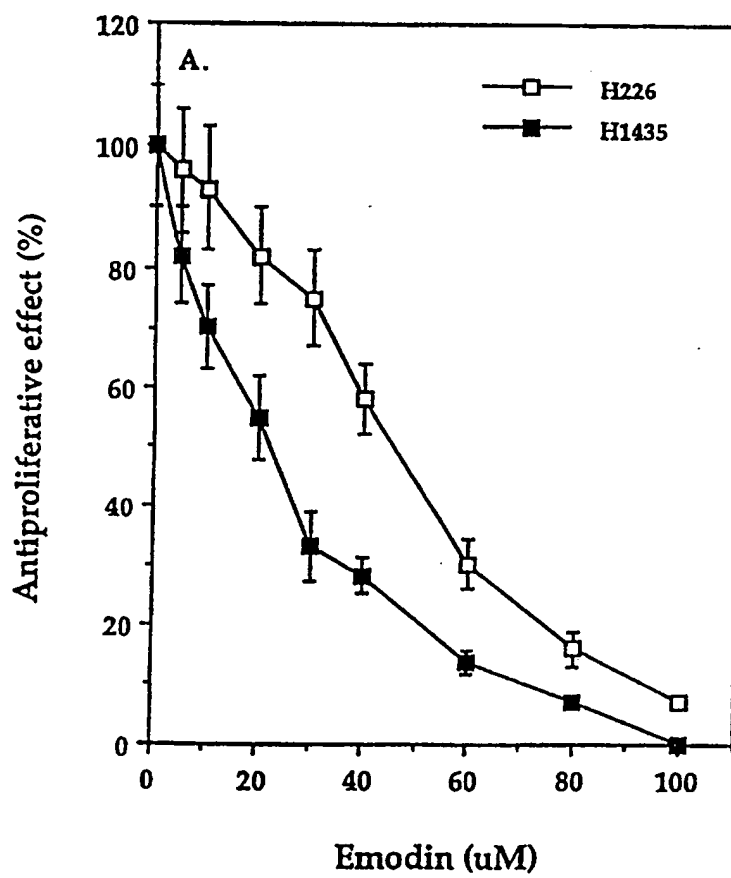


FIG. 7A

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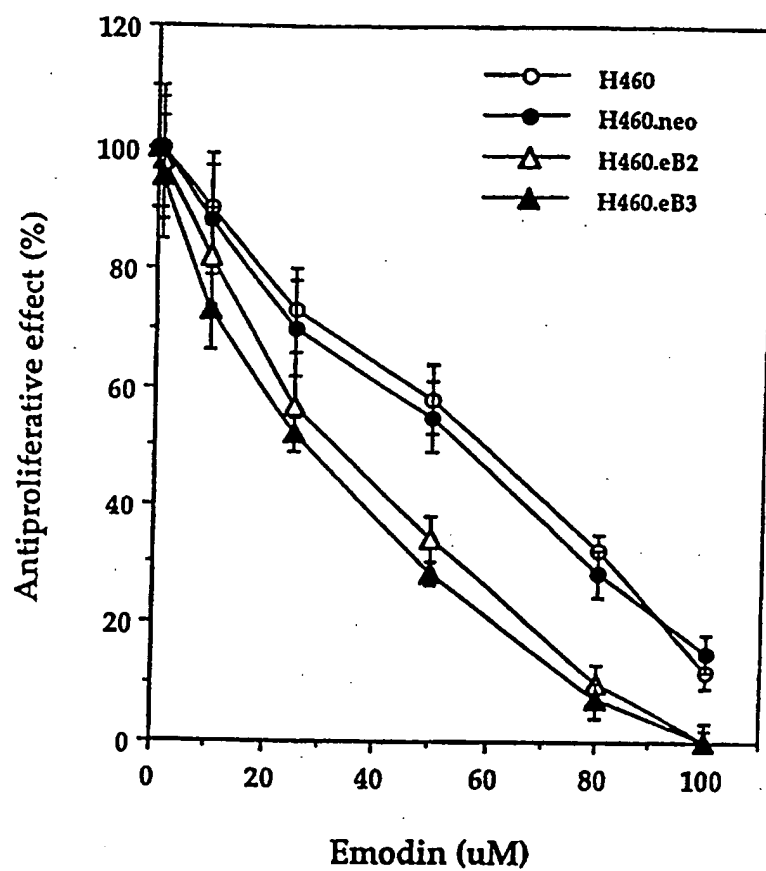


FIG. 7B

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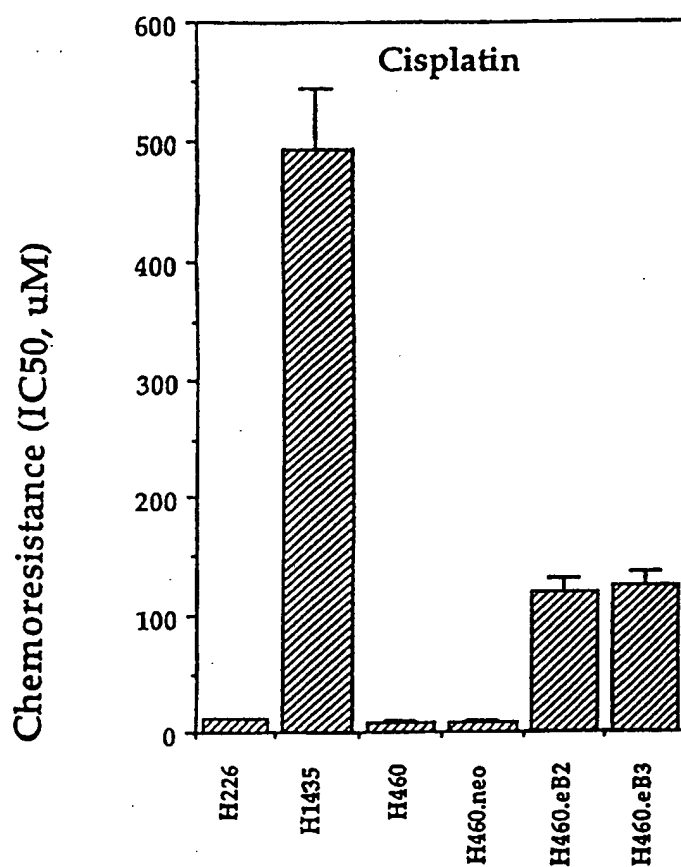


FIG. 8A

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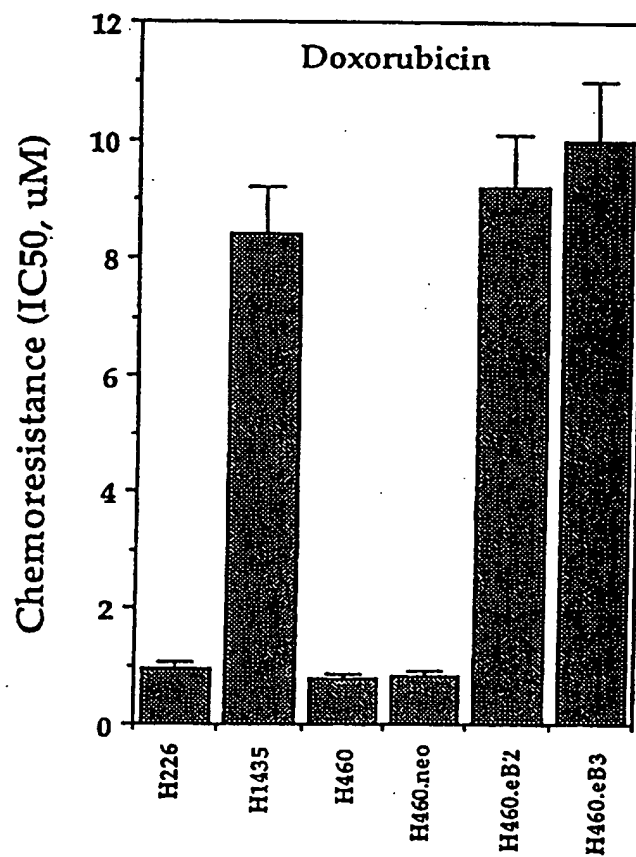


FIG. 8B

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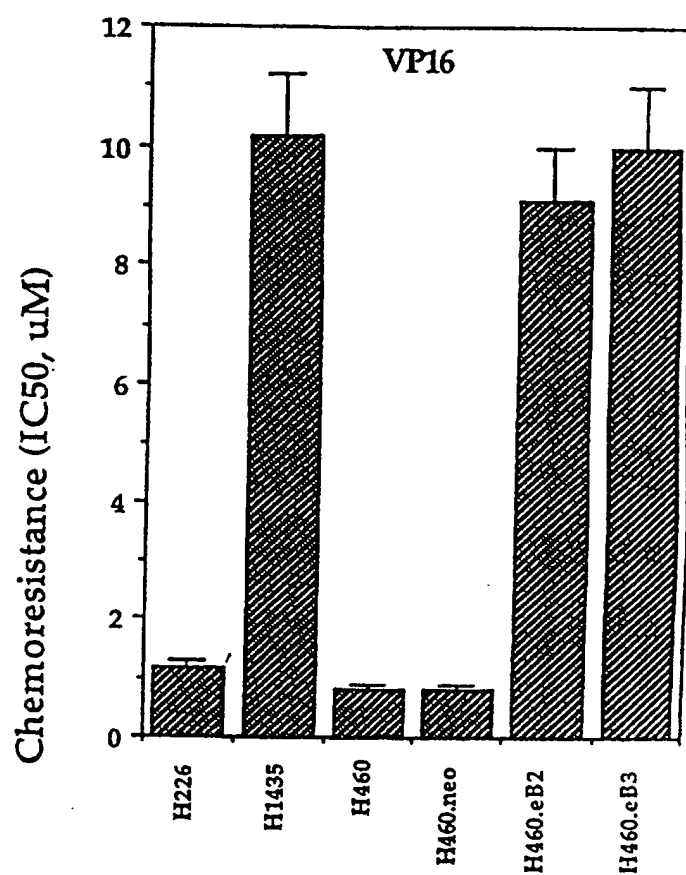
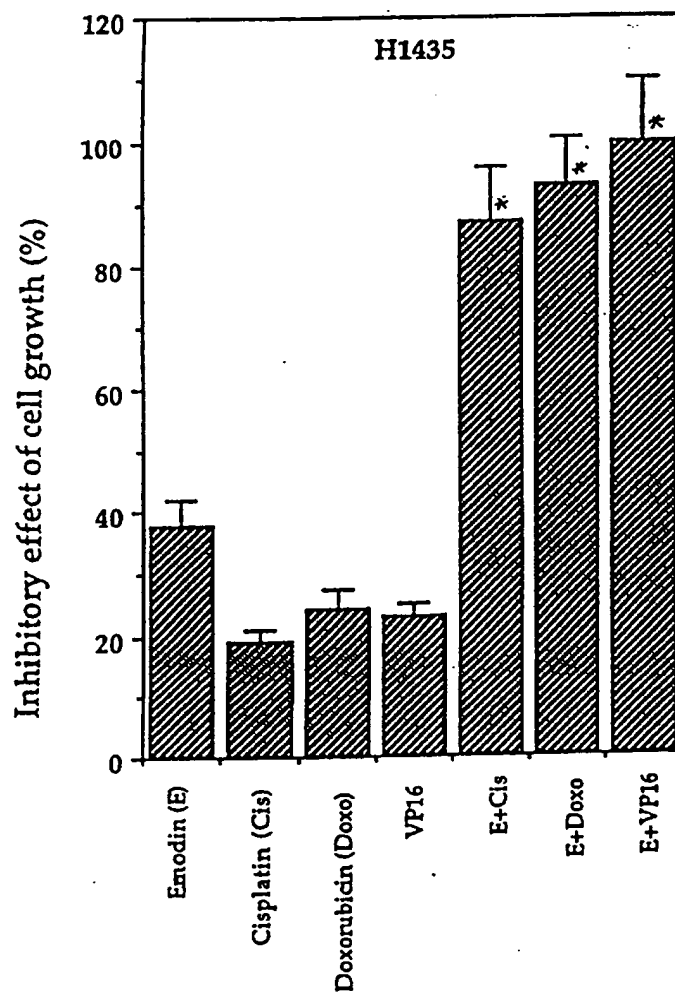


FIG. 8C

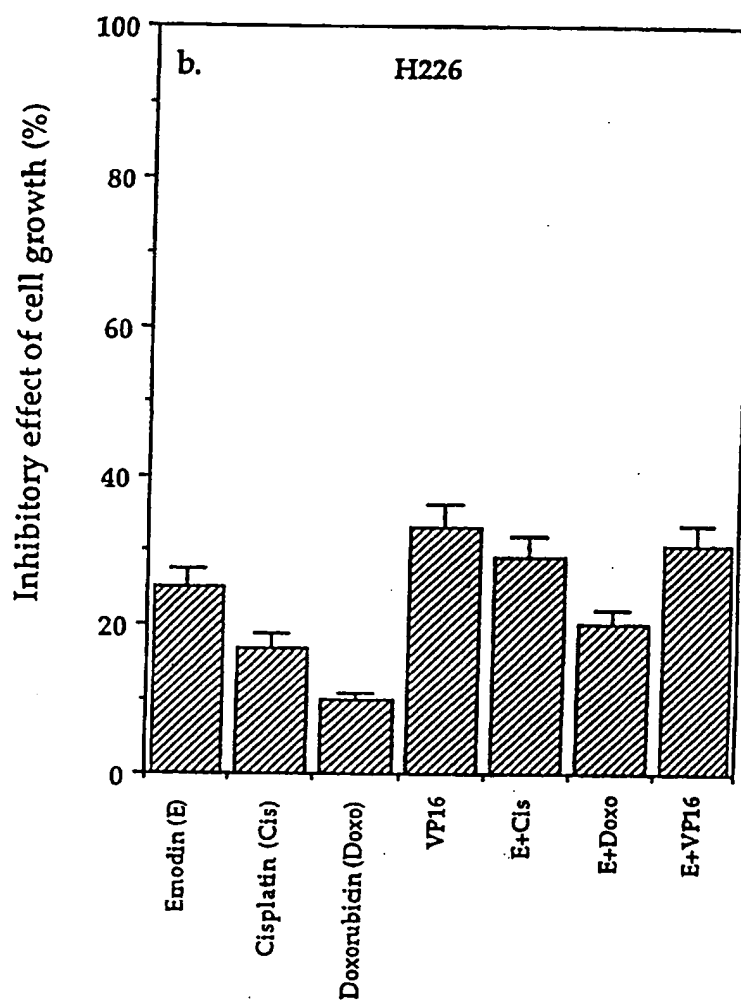
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FIG. 9A



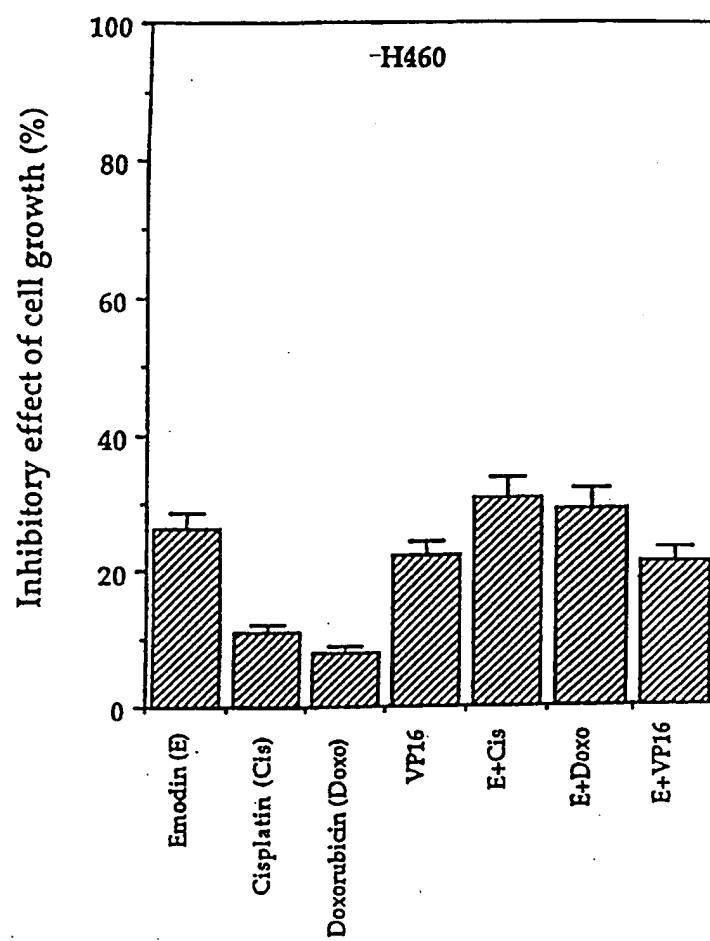
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FIG. 9B



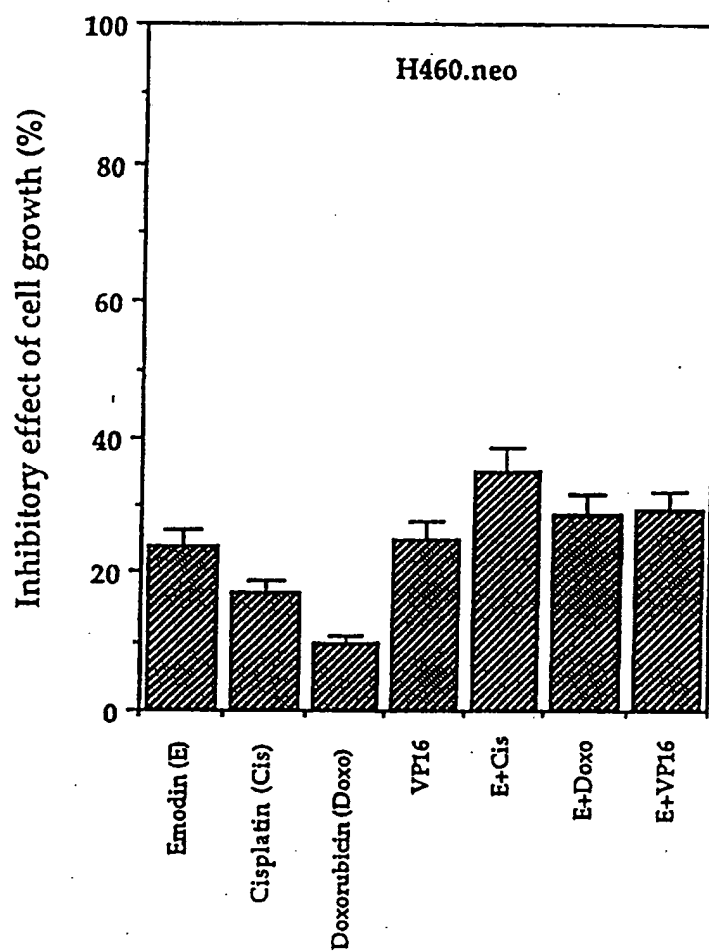
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FIG. 9C



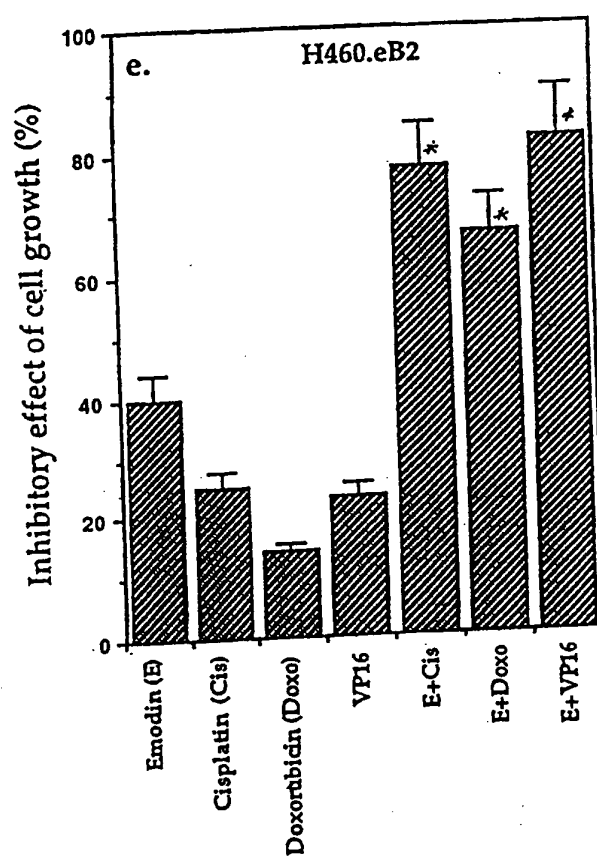
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FIG. 9D



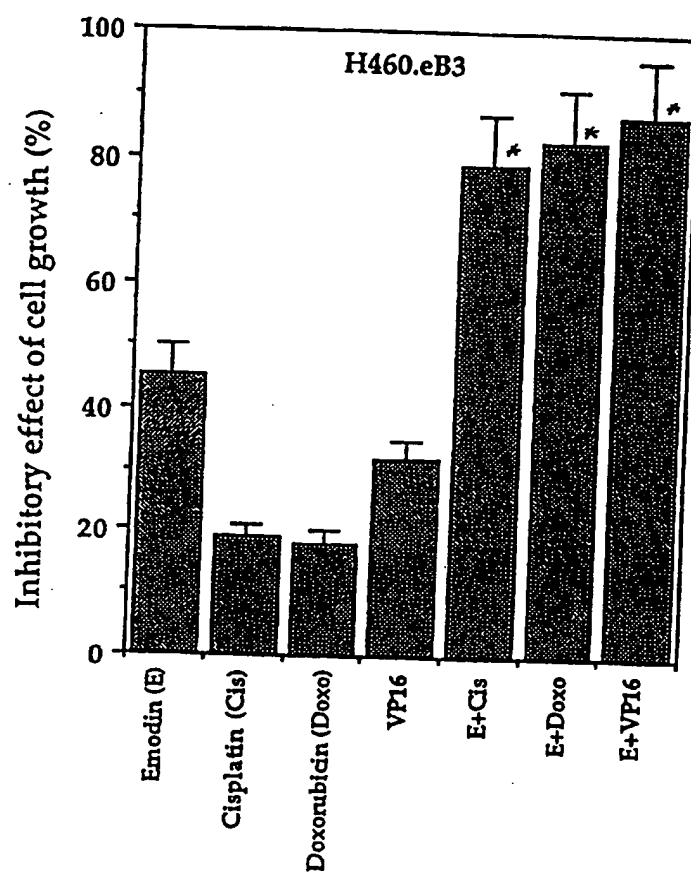
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FIG. 9E



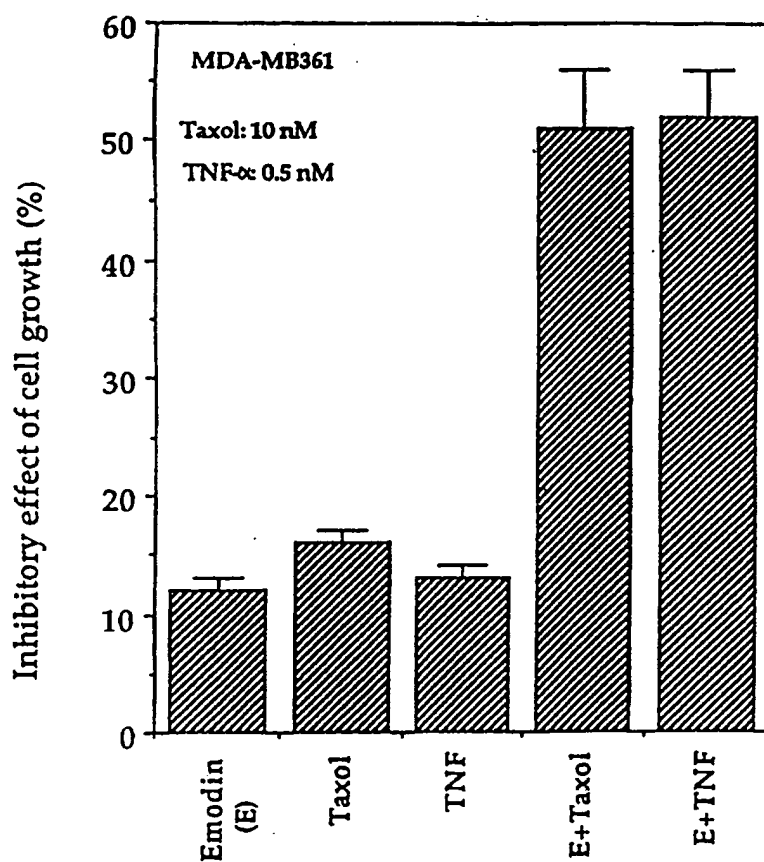
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FIG. 9F



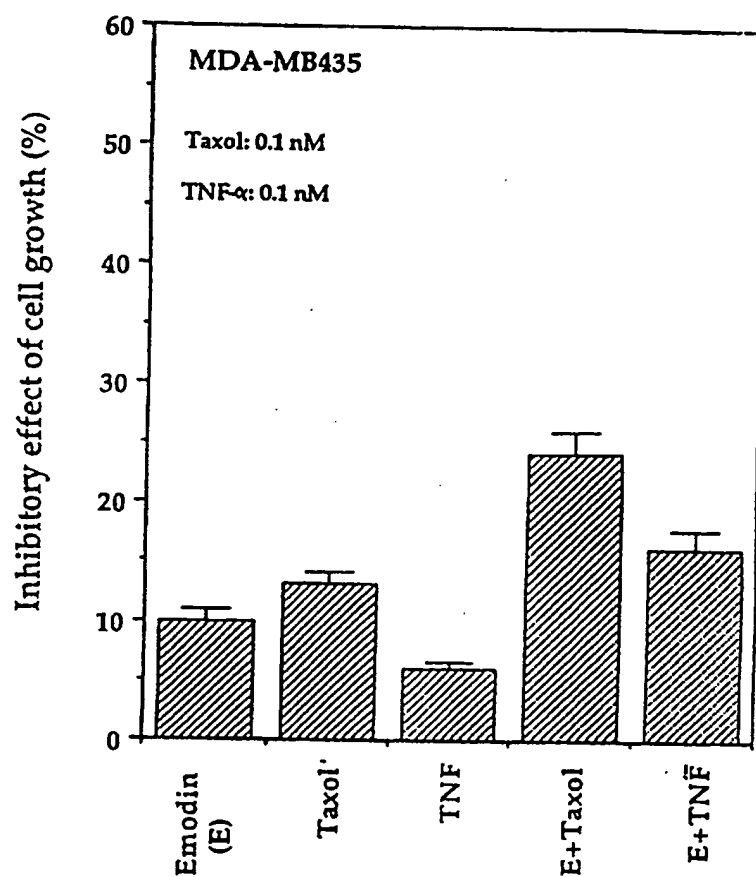
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FIG. 9G

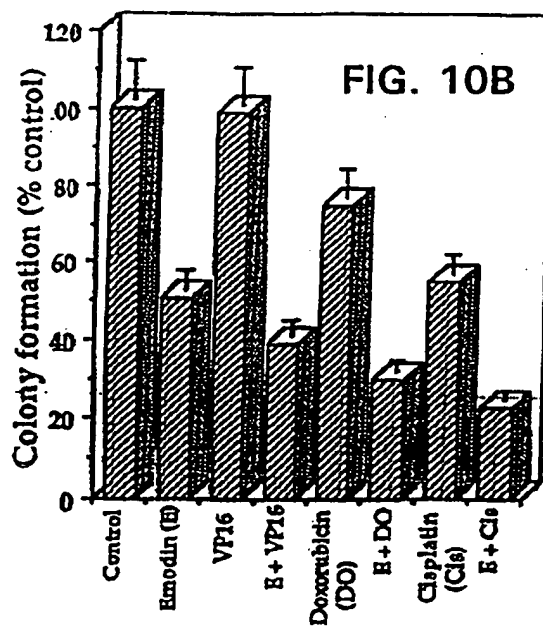
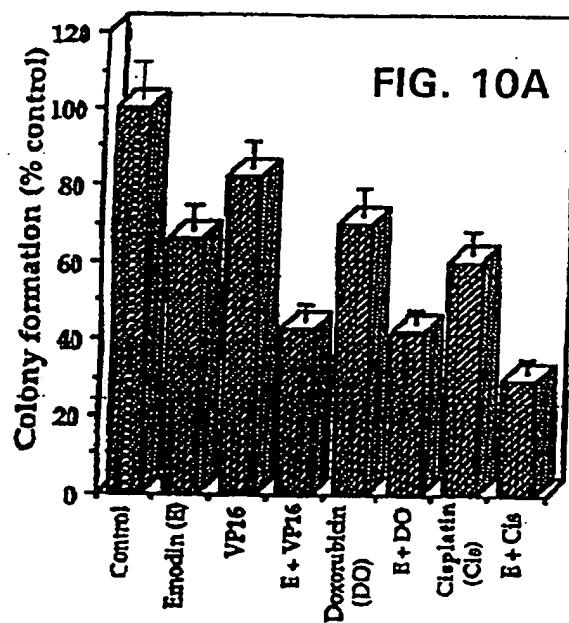


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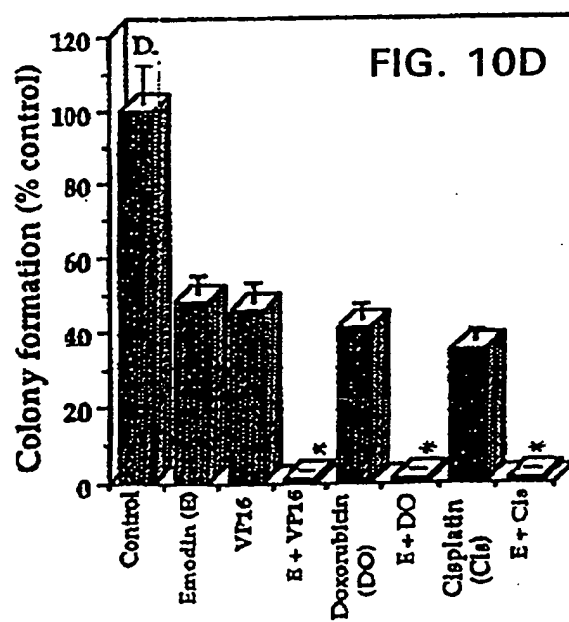
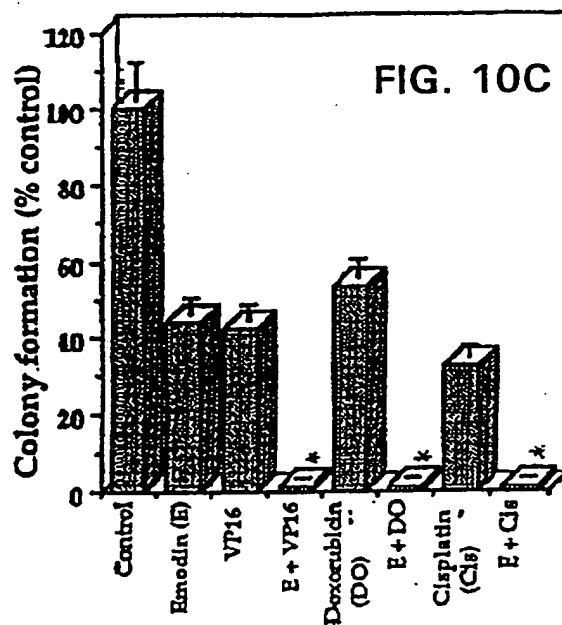
FIG. 9H



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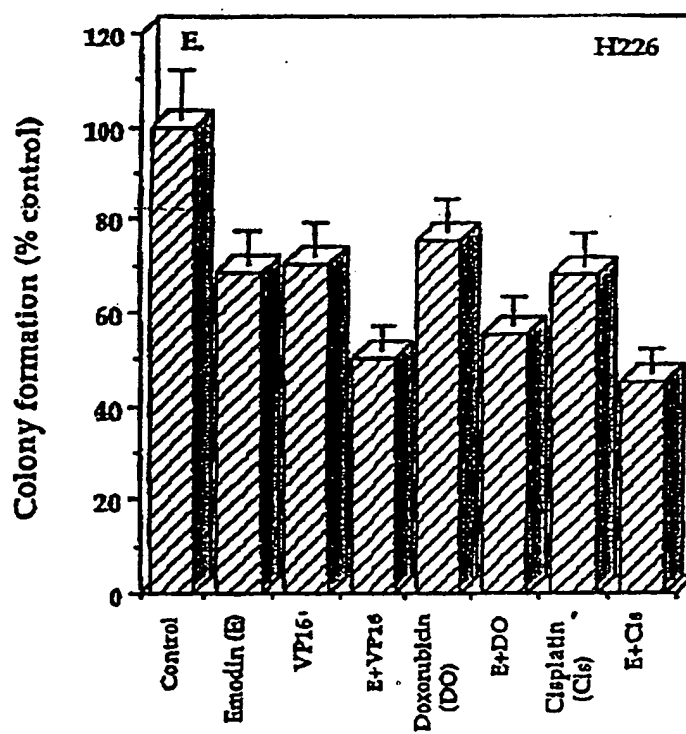


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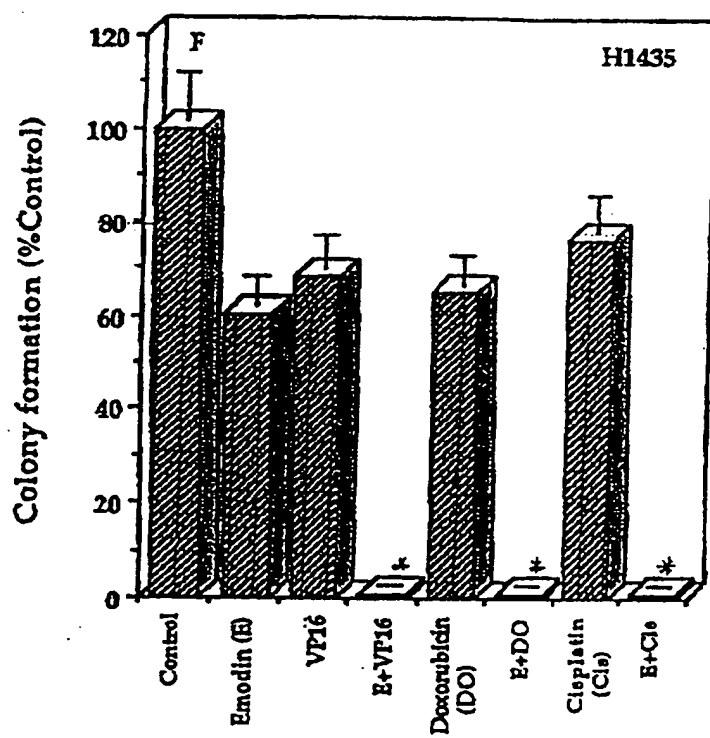
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FIG. 10E



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FIG. 10F



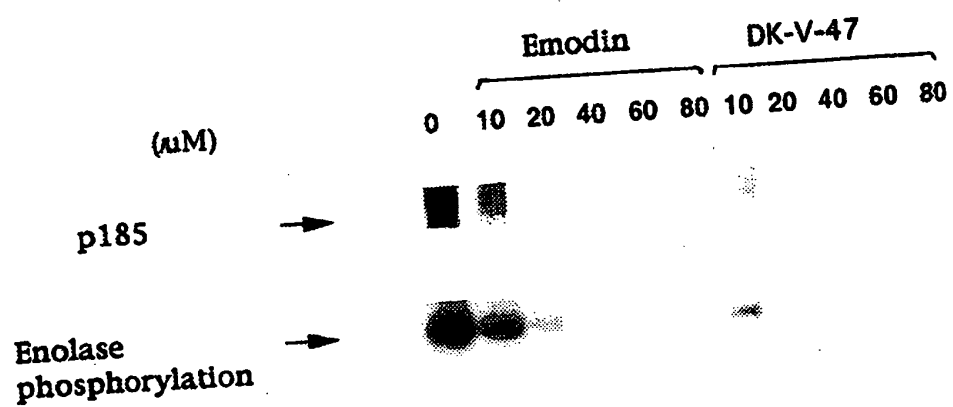


FIG. 12

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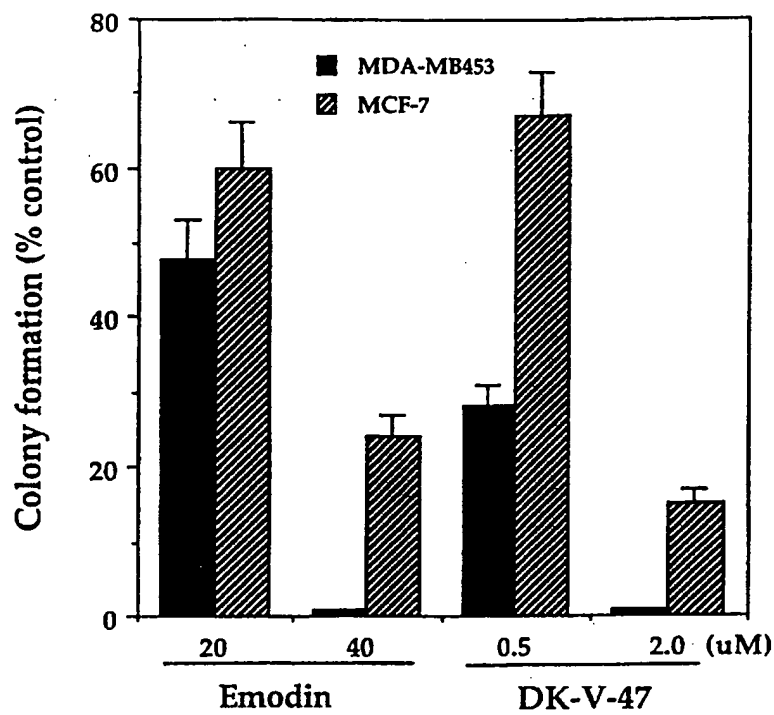


FIG. 11A

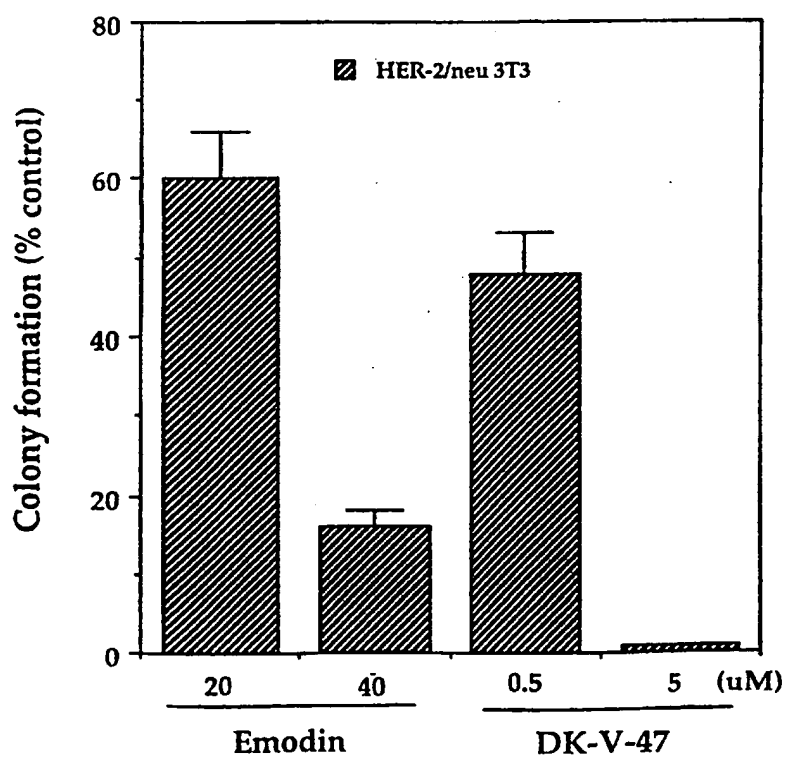


FIG. 11B

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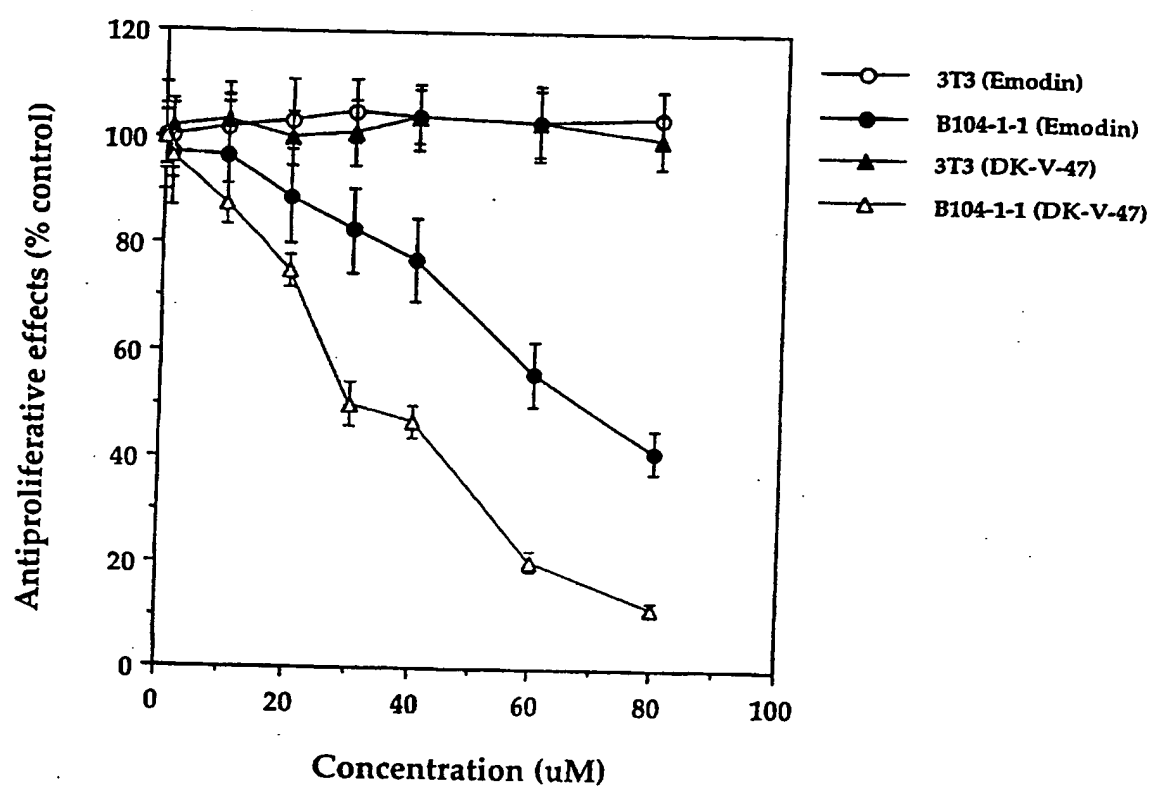


FIG. 13

FIG. 14A.

A.

DK-V-47 (μM) 0 10 20 40 60 80

(kDa)

92 →

68 →

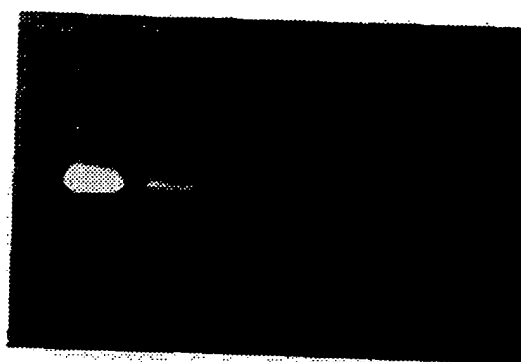


FIG. 14B.

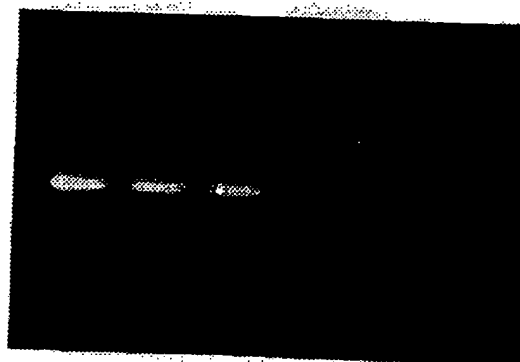
B.

Emodin (μM) 0 10 20 40 60 80

(kDa)

92 →

68 →



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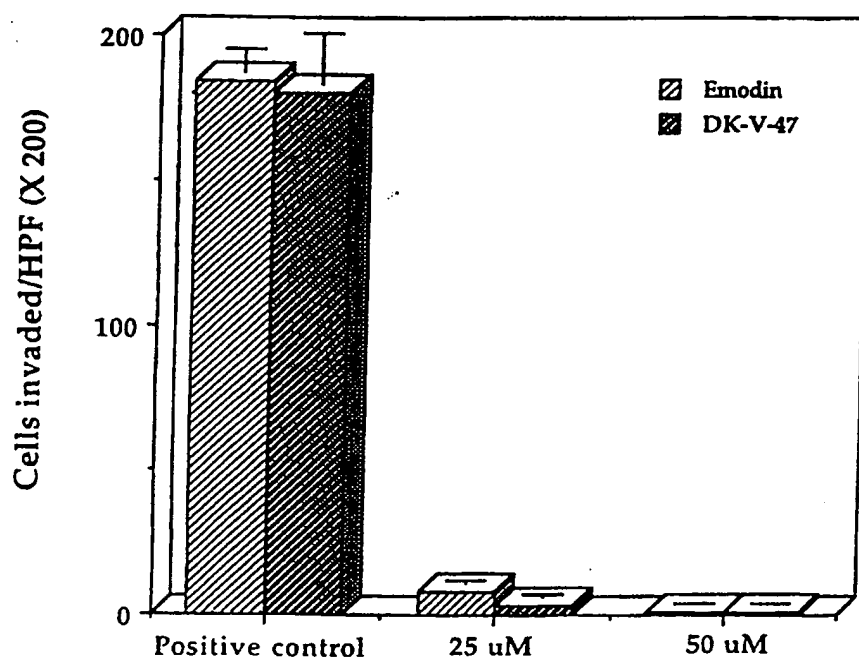


FIG. 15

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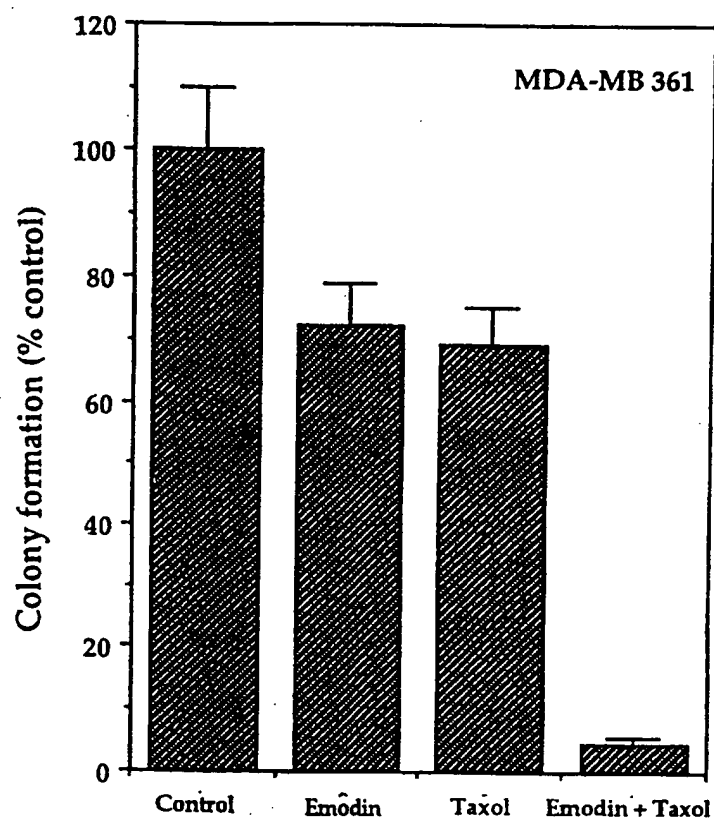


FIG. 16A

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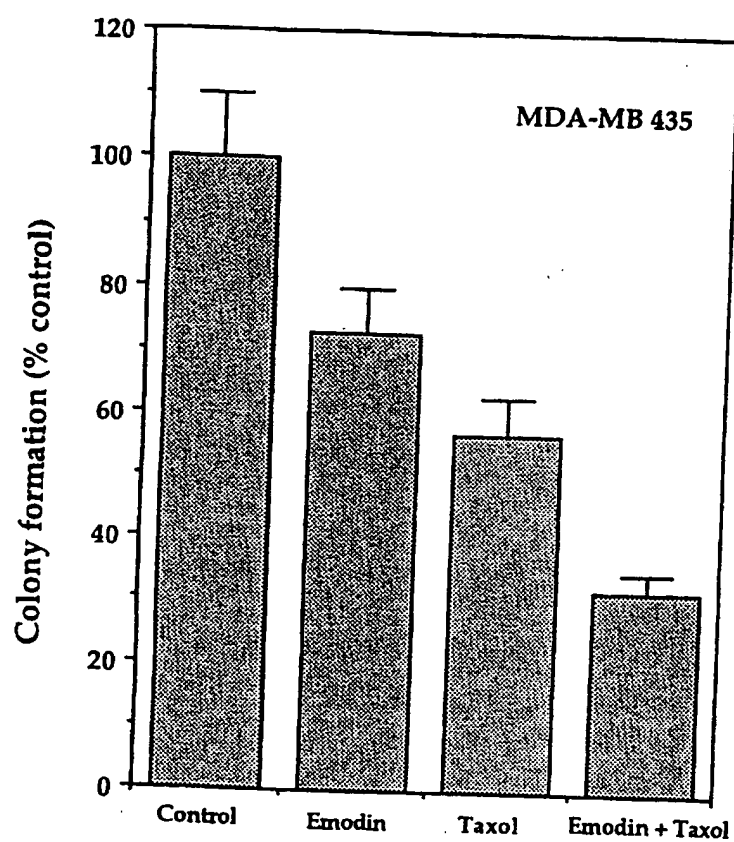


FIG. 16B

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FIG. 17A

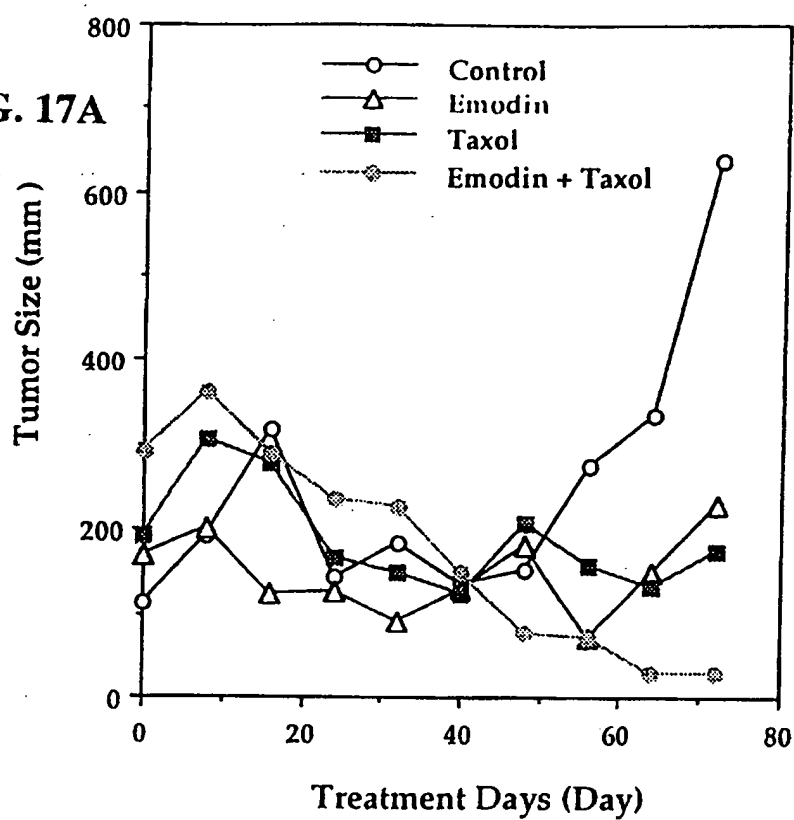
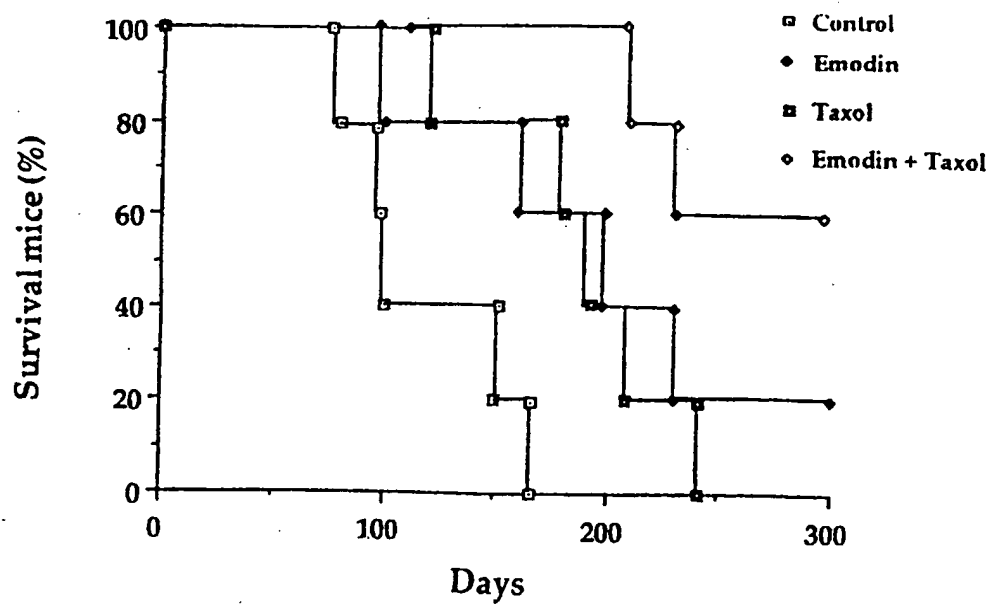


FIG. 17B



INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 97/01686

| A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K31/12 | | | | |
|--|---|--|---|---|
| According to International Patent Classification (IPC) or to both national classification and IPC | | | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K | | | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. | | |
| X | CANCER RESEARCH, vol. 55, - 1995 pages 3890-3896, XP000673653 ZHANG, CHANG, BACUS, HUNG: "SUPPRESSED TRANSFORMATION AND INDUCED DIFFERENTIATION OF HER-2/NEU OVEREXPRESSING BREAST CANCER CELLS BY EMODIN" cited in the application | 1-3,5-8, 14,19, 24,26, 27,45, 46,48-52 | | |
| A | see page 3890, right-hand column, paragraph 1; figures 1-4 | 28,29, 31,34 | | |
| A | see page 3893, left-hand column, paragraph 3 - right-hand column, paragraph 1 see page 3895, left-hand column --- -/-- | 36-39 | | |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. | | | | |
| <input checked="" type="checkbox"/> Patent family members are listed in annex. | | | | |
| * Special categories of cited documents : <table border="0"> <tr> <td style="vertical-align: top;"> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="vertical-align: top;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "d" document member of the same patent family </td> </tr> </table> | | | "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "d" document member of the same patent family |
| "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "d" document member of the same patent family | | | |
| Date of the actual completion of the international search 27 May 1997 | | Date of mailing of the international search report 09.06.97 | | |
| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | | Authorized officer Kanbier, D | | |

INTERNATIONAL SEARCH REPORT

Intern: I Application No
PCT/US 97/01686

| C.(Continuation) DOCUMENTS C NSIDERED TO BE RELEVANT | | |
|--|--|---|
| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| X | WO 95 13813 A (UNIV DUKE) 26 May 1995 see page 19, paragraph 2 - page 21, paragraph 4; claims 1,3,6,8-10,13-15,18,19; example 5 see page 2, paragraph 3 - page 3, paragraph 2; tables 1-4 see page 6, line 1-2 see page 14, paragraph 1 --- | 1,7, 11-19, 21-24, 26-30, 33-35, 37,38, 40-44 |
| X | WO 90 08759 A (SLOAN KETTERING INST CANCER) 9 August 1990 see page 4-7; claims 1,9,10,12; tables 1-4 --- | 27,45,49 |
| A | see page 4-7; claims 1,9,10,12; tables 1-4 --- | 21,47, 48,50-52 |
| X | BIOLOGICAL CHEMISTRY HOPPE-SEYLER, vol. 373, 1992, pages 903-910, XP000672358 JINSART ET AL: "INHIBITION OF MYOSIN LIGHT CHAIN KINASE, CAMP-DEPENDENT PROTEIN KINASE, PROTEIN KINASE C AND OF PLANT CA-DEPENDENT PROTEIN KINASE BY ANTHRAQUINONES" see page 908, right-hand column - page 909; table 1 --- | 27,45, 47-50 |
| A | see page 908, right-hand column - page 909; table 1 --- | 1,4-6, 21,34, 36-39 |
| X | PLANTA MEDICA, vol. 54, 1988, pages 413-414, XP000672231 YEH ET AL: "EFFECTS OF ANTHRAQUINONES OF POLYGONUM CUSPIDATUM ON HL-60 CELLS" see page 413 - page 414; table 1 --- | 27,45, 48-50 |
| A | see page 413 - page 414; table 1 --- | 1,4-6, 24,34, 36-39,47 |
| P,X | ONCOGENE, vol. 12, 1996, pages 571-576, XP000672224 ZHANG, HUNG: "SENSITIZATION OF HER-2/NEU-OVEREXPRESSING NON-SMALL CELL LUNG CANCER CELLS TO CHEMOTHERAPEUTIC DRUGS BY TYROSINE KINASE INHIBITOR EMODIN" cited in the application see figures ----- | 1-3,5-8, 14-19, 24-27, 34-41, 45,46, 48-52 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/01686

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 28-33, 20
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☒ Claims Nos.: 1-19, 21-27, 45-52
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
The wording of the claims mentioned below does not make it clear if "cells"
are located in animal bodies or if they are only cell cultures in vitro. In
the former case, the subject matter of these claims would not be patentable
for the same reason as for claims 20, 28-33 (see above)
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Intern: I Application No
PCT/US 97/01686

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO 9513813 A | 26-05-95 | US 5436243 A | 25-07-95 |
| | | AU 1098995 A | 06-06-95 |
| ----- | | | |
| WO 9008759 A | 09-08-90 | US 4966918 A | 30-10-90 |
| | | US 5053431 A | 01-10-91 |
| ----- | | | |